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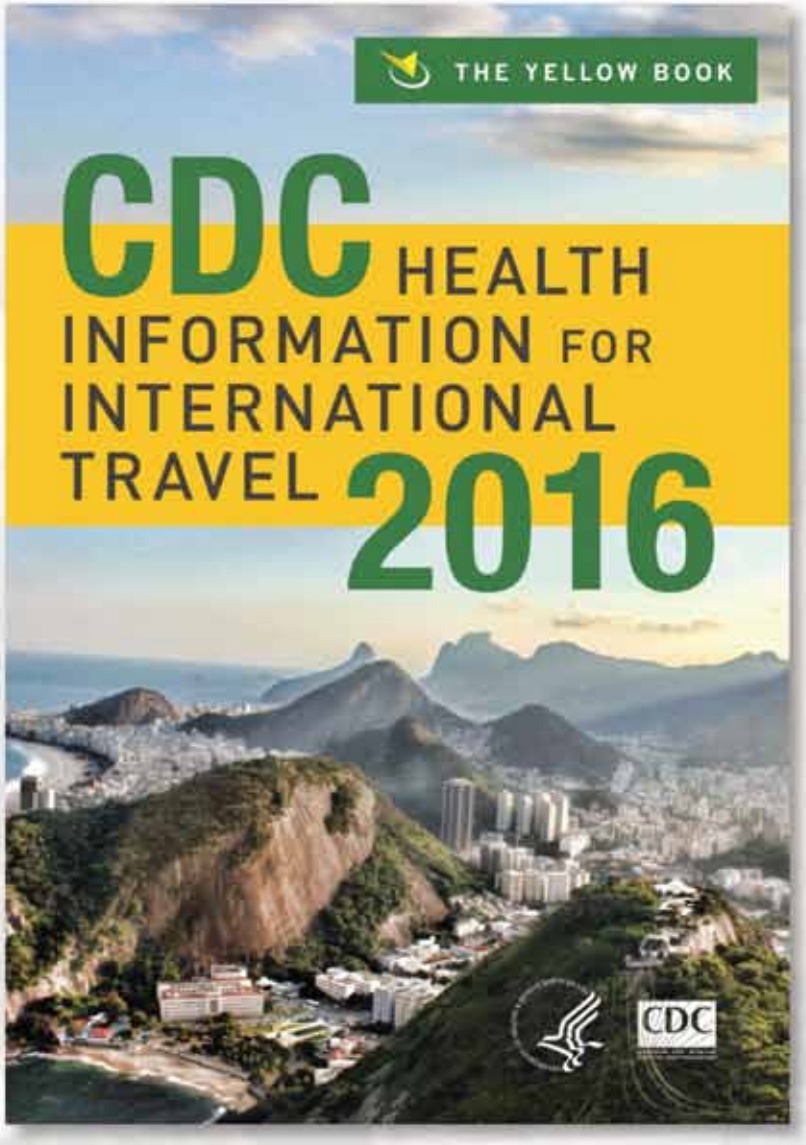

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An Operational Framework for Insecticide Resistance Management Planning

Emmanuel Chanda,[1] Edward K. Thomsen,[1] Mulenga Musapa, Mulakwa Kamuliwo, William G. Brogdon, Douglas E. Norris, Freddie Masaninga, Robert Wirtz, Chadwick H. Sikaala, Mbanga Muleba, Allen Craig, John M. Govere, Hilary Ranson, Janet Hemingway, Aklilu Seyoum, Michael B. Macdonald, Michael Coleman

Arthropod vectors transmit organisms that cause many emerging and reemerging diseases, and their control is reliant mainly on the use of chemical insecticides. Only a few classes of insecticides are available for public health use, and the increased spread of insecticide resistance is a major threat to sustainable disease control. The primary strategy for mitigating the detrimental effects of insecticide resistance is the development of an insecticide resistance management plan. However, few examples exist to show how to implement such plans programmatically. We describe the formulation and implementation of a resistance management plan for mosquito vectors of human disease in Zambia. We also discuss challenges, steps taken to address the challenges, and directions for the future.

Emerging and reemerging infectious diseases are often transmitted by arthropod vectors (*1,2*). A primary strategy to reduce vectorborne disease transmission is the use of insecticides for public health. However, resistance to insecticides has appeared in all major insect vectors of human disease (*3*) and has rapidly increased in prevalence and intensity over the past decade (*4*). Insecticide resistance is worrisome, in part because it has repeatedly been implicated as a cause of disease resurgence, particularly for malaria (*5–8*).

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Although the spread of resistance in a population of organisms challenged by a drug or insecticide is inevitable, the public health community has not yet taken the steps necessary to safeguard the limited number of insecticides available. Consequently, their continued efficacy is at risk (*9*), setting the stage for reemergence of vectorborne diseases in locations where insecticide-based control measures are implemented. In response to this concern, the World Health Organization published the Global Plan for Insecticide Resistance Management (GPIRM) in 2012 (*10*). This document provides vectorborne disease control programs and other stakeholders with a strategic direction for approaching the resistance crisis.

The GPIRM outlines the need for increased resistance monitoring, data management capacity, and implementation of strategies to manage insecticide resistance. The responsibility for implementing this management plan lies with disease-endemic countries, with support from their global partners. Among the challenges in carrying out these recommendations are lack of options for insecticide rotations (the primary strategy for curbing spread of resistance), inconsistent resistance monitoring procedures, reluctance to share resistance data, and lack of data management capacity in disease-endemic countries (*11*). In addition, a reliance on donor funding, which can be unpredictable, threatens the sustainability of enacted plans.

By 2014, only a few countries had established insecticide resistance management plans (IRMPs) and incorporated them into operational malaria vector control programs (*11*). However, no country has documented how it formulated or executed these policies and addressed challenges. To realize fully the vision of the GPIRM, national malaria control programs must share experiences regarding policymaking processes. We report on the formulation and implementation of a new insecticide resistance management plan in Zambia during 2009–2014.

[1]These authors contributed equally to this article.

Local Setting

Because of the increased political commitment since the Abuja Declaration in 2001, funding for malaria control has increased (*12*). The widespread use of insecticide-based vector control subsequently reemerged in many malaria-endemic countries, including Zambia. In 2000, the private sector reintroduced indoor residual spraying (IRS) with 2 classes of insecticides, DDT and pyrethroids, in 2 districts in Zambia's Copperbelt province (*13*). The success of these IRS programs led Zambia's National Malaria Control Centre (NMCC) to implement IRS and distribution of long-lasting insecticidal nets (LLINs) (*14*). IRS use was scaled up from 5 districts in 2003 to all 72 districts by 2011 (*15*) (the total number of Zambia districts increased to 105 in 2013). Countrywide mass distribution of LLINs has occurred since 2005; currently, 72% of households own ≥1 LLIN (*16*). For 9 years after IRS was introduced in 2000, vector control relied exclusively on 2 insecticide classes with 1 mode of action (i.e., both DDT and pyrethroids target the voltage-gated sodium channel of nerve cells to cause death of the insect). However, in 2009, resistance to both insecticide classes was detected in the major malaria and lymphatic filariasis vectors (*17*).

After this resistance was detected, the NMCC formed the Insecticide Resistance Management Technical Working Group (IRMTWG) in 2010. This group provides a foundation for the policy development process by coordinating policy formulation, implementation, and evaluation. The IRMTWG is multisectoral and comprises members of government, nongovernment, and private organizations having a vested interest in vector control (Figure 1). A subset of IRMTWG members forms the Technical Advisory Committee (TAC), which aids the NMCC in interpreting the results of the implementation process and provides recommendations about the most appropriate actions. The NMCC serves as the secretariat of the IRMTWG and coordinates the implementation of entomologic surveillance and resistance monitoring in the country.

In April 2011, through a series of conference calls, the IRMTWG defined the following policy objectives: 1) gather more phenotypic resistance data through partners serving as channels of information for different parts of the country, and establish a sustainable monitoring protocol; 2) determine the underlying mechanisms involved in resistance of all the major vectors in Zambia; 3) establish and maintain a database for all insecticide resistance data; and 4) develop an IRMP informed by data and agreed on by all stakeholders. The group recognized that to achieve the last objective, objectives 1–3 must be first realized. Therefore, implementation of the policy was envisioned as a multiphased process.

Objective 1: Expand Resistance Data

Although initial data presented to the incipient IRMTWG were limited, they highlighted insecticide resistance in Zambia, in particular, DDT resistance in areas where this insecticide was used (*17*). In addition, 2 obstacles became apparent. First, the available data revealed only a small portion of the insecticide resistance in the country, but the data could be expanded through the collaboration of other stakeholders, including research (local and international), private (mining and agricultural), nongovernment

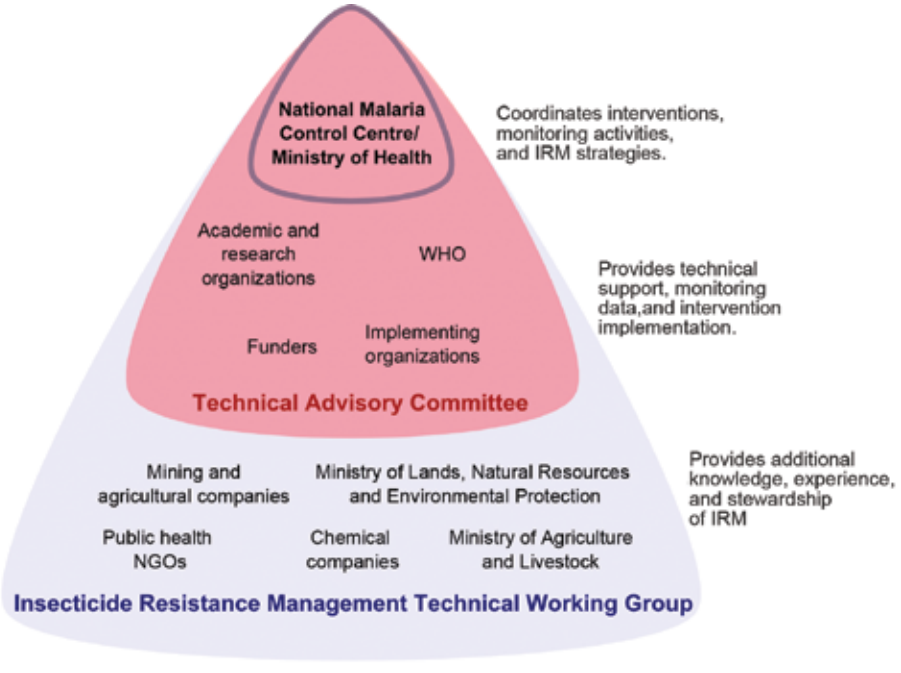

Figure 1. Composition of the Insecticide Resistance Management (IRM) Technical Working Group and the Technical Advisory Committee in Zambia and roles of member organizations. NGOs, nongovernment organizations; WHO, World Health Organization.

organizations, and insecticide companies. Second, even if all partners agreed to share data, a concerted and organized effort to collect more data was necessary.

The formation of the IRMTWG and its inclusion of all stakeholders assuaged the doubts of many of its participants about sharing data. Discussions about data ownership and use continue to the present day, and all participants agree that data-sharing does not prevent publication. In addition, partners are willing to share data because of the NMCC's role in leading the process. Their willingness to share data might not have occurred if the process were led by an outside organization. With data from the entire country in hand, the IRMTWG and TAC could make informed recommendations about which locations needed additional data and which organization was in the best position to collect that data.

As a result of data-sharing and increased monitoring efforts, the cumulative number of geographic foci with resistance data increased at a staggering pace (Figure 2), largely because of efficient interactions between the IRMTWG and policy implementers (i.e., the NMCC and the Zambia Integrated Systems Strengthening Program, supported by the US government's President's Malaria Initiative [PMI]). During the annual meeting, the IRMTWG collates and interprets the available resistance data and provides guidance about locations for focusing monitoring efforts for the following year. This work has resulted in a better understanding of the resistance profiles of the major malaria vectors throughout the country.

Sustained Monitoring and Evaluation

The insecticide resistance profile for many areas of the country was determined, each at a specific time, during 2011–2014. However, 9 sites in districts where IRS was being implemented were selected for longitudinal monitoring of mosquito populations. In these sites, light traps (developed by the US Centers for Disease Control and Prevention [CDC]) and pyrethrum spray catches were used to monitor mosquito density and behavior; insecticide bioassays (*18*) were performed every 6 months to monitor prevalence of resistance. In 2014, the IRMTWG decided that the NMCC should increase the number of sites being monitored from 9 to 24 sentinel sites spread across the country. When resistance is detected by using a diagnostic dose, quantifying the strength of that resistance and determining how the resistance may impact vector control are needed. In Burkina Faso, the strength of resistance increased significantly over just 3 years (*19*), leading to decreased effectiveness of bed nets. Consequently, CDC bottle bioassays (*20*) will be used to measure intensity of resistance in the original 9 sentinel sites.

The primary challenge in implementing this monitoring scheme is the limited financial and human resources

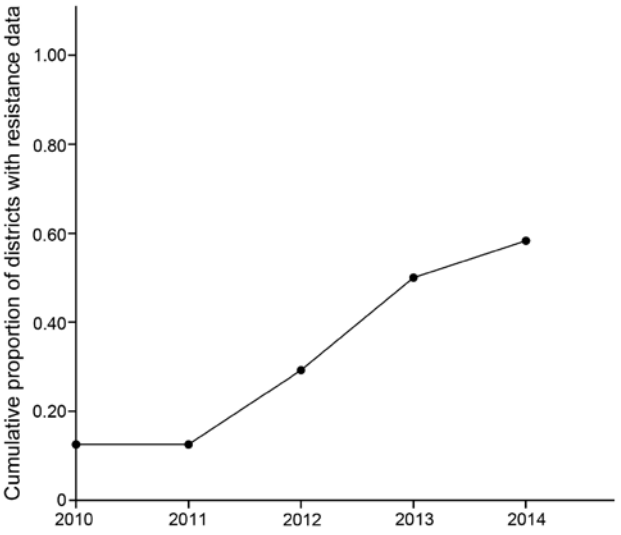

Figure 2. Increase in the number of geographic locations producing data on insecticide resistance in malaria vectors, Zambia, 2010–2014.

that any single organization has to devote to such a large task. This difficulty was alleviated when the NMCC took the lead in the insecticide resistance management process. Although funding comes from different sources (e.g., government of Zambia, World Health Organization, PMI, UK Department for International Development, and the Global Fund), having the NMCC at the helm helps divide monitoring procedures among partners in an organized fashion and facilitates the standardization of monitoring protocols across sites.

Objective 2: Determine Mechanisms of Resistance

With development of molecular techniques for resistance detection, programs can now detect the presence of resistance genes in vectors and monitor how gene frequencies change over time. Both Equatorial Guinea (*21*) and Zambia (*22*) have shown the value of using rapid molecular assessment of local vectors to inform operational decision-making.

Details of insecticide resistance in Zambia and the means of data collection have been documented (*17,22*). Briefly, *Anopheles gambiae* mosquitoes are resistant to DDT and pyrethroids because of the target site mutation *kdr* (knockdown resistance) L1014F and elevated P450 and glutathione S-transferase detoxification enzymes; *An. funestus* mosquitoes are resistant to carbamates and pyrethroids because of elevated P450. In *An. funestus* mosquitoes, this resistance mechanism is the same one that led to the failure of the pyrethroid-based control program in South Africa during the 1990s (*23*) and that has been detected in Malawi (*24*) and Mozambique (*25*). These findings greatly influenced the decision-making process

surrounding the IRM strategy in Zambia; they confirmed that pyrethroids should no longer be included in insecticide rotations because of the metabolic mechanism of resistance (*10*).

Objective 3: Establish a Central Data Repository

Reliable and available resistance-monitoring data are essential for evidence-based decision-making. The Disease Data Management System (DDMS) entomology module (*26*) is used for collating the data, and evaluation of the DDMS in Zambia and other countries has been reported (*27*). Historical data since 2011 were imported into the DDMS from legacy systems (typically Excel or Access [Microsoft, Redmond, WA, USA]), and data from ongoing collections have been entered into the system directly. The DDMS accepts all data used by vectorborne disease control programs and generates reports in many formats. When evaluating resistance data, the DDMS has become a valuable tool for the IRMTWG and TAC. Information is more easily collated and mapped than previously and enables more informed decision-making and close monitoring of the IRM policy development process.

The primary challenge in adopting a system like the DDMS has been a lack of data management capacity within the NMCC. Components of entomologic monitoring and evaluation, such as data management and decision support systems like the DDMS, must have allocations of sufficient financial and human resources if they are to realize their full potential in guiding disease control policy. The NMCC and the Zambia Integrated Systems Strengthening Program sought technical assistance for training end users, from data entry to management levels, so that all parties were competent in using the system. The annual IRMTWG meetings that require access to the data and reports generated from the DDMS provide pressure to use the system (i.e., enter and check the data) throughout the year.

Objective 4: Policy Change and the IRMP

The primary goal of the IRMTWG annual meetings is to interpret resistance data to make informed decisions about which insecticides to deploy for IRS during the next spray season. During 2011–2013, when implementing partners were strategically collecting resistance data to better inform the IRM strategy, the TAC had to make decisions about IRS in Zambia, although some parts of the country were not comprehensively supported by data. In 2011, the first major change to the IRS strategy was implemented: withdrawing DDT and introducing carbamates and organophosphates. By using the resistance data available at the time, TAC decided that carbamates should be deployed in Northern, Muchinga, Luapula, and Copperbelt provinces, whereas organophosphates would be used in Eastern province and pyrethroids in the rest of the country. After the necessary legislative and regulatory approvals were obtained from the Zambia Environmental Management Agency, procedures for procuring these insecticides immediately commenced. Both the PMI and the government of Zambia, which supported the IRS operations, issued bids for contracts, and contending bidders registered with the Zambia Environmental Management Agency as a regulatory compliance procedure for supplying carbamates and organophosphates. To operationalize the new policy, personnel in public and private sectors conducted train-the-trainer workshops and cascade trainings that emphasized safe use, storage, and disposal of carbamates and organophosphates (*28*).

In 2012, the TAC reviewed the latest data from all IRMTWG member organizations and advised the NMCC to rotate organophosphates with carbamates in Northern, Muchinga, and Copperbelt provinces for the 2012 IRS campaign. The same insecticides used in 2011 were recommended in the other provinces because of lack of available options or lack of data to support decisions. Unfortunately, this strategy was not executed, and Northern, Muchinga, and Copperbelt provinces were sprayed with carbamates again in 2012 because of an insufficient amount of the new formulation of the organophosphate pirimiphos-methyl, Actellic 300CS (Syngenta, Basil, Switzerland), being produced by the supplier. In addition, because of the paucity of data in the western half of the country, the TAC advised that efforts should be made to collect resistance data in these areas.

In 2013, efforts to collect resistance data in North-Western and Western provinces were increased to better inform decision-making. The available data indicated that resistance to all classes of insecticides except the organophosphates was present in all areas of Zambia. Therefore, the TAC recommended that the NMCC spray the entire country with the organophosphate pirimiphos-methyl in 2013. However, only Luapula, Northern, Muchinga, and Eastern provinces were sprayed in 2013 because of increased cost. The TAC recommended continued monitoring of resistance across the country, with additional efforts in Luapula province, where resistance patterns to pyrethroids appeared to be inconsistent.

With the resistance profile in all areas of the country established, the insecticide resistance database in use, and regular IRMTWG and TAC meetings being held, the culmination of several years of hard work was evident in 2014. In preparation for the annual IRMTWG and TAC meeting in May, the first official IRMP was drafted and distributed to members in March 2014. Over the course of the meetings, the IRMP was revised and modified, resulting in a living document that will guide the control program about IRM strategies, monitoring and evaluation, and operational research priorities (Zambia Ministry of Health, unpub. data).

The plan highlights the necessity of avoiding pyrethroids for IRS because of widespread, metabolically mediated resistance. In addition, it recommends that organophosphates be used in rotation with DDT (where *An. funestus* or *An. arabiensis* mosquitoes are the primary vectors) to control vector populations effectively while simultaneously reducing selection pressure of any specific active ingredient. The plan highlights several knowledge and resource gaps, including the limited number of insecticides available for IRS, the limited human resource and institutional capacity to deliver monitoring and evaluation, and limited data available to better target IRS interventions.

The primary challenge in developing the IRMP has been a lack of options for insecticide rotation. Ideally, insecticides that have different modes of action should be alternated or used in a mosaic to reduce selection pressure. However, only 4 classes of insecticides with 2 modes of action are currently recommended for IRS. The primary vectors in Zambia have different cross-resistance patterns, and their ranges overlap throughout much of the country, making the choice for control limited in an insecticide-based program. Industry must continue to develop new classes of insecticides that can be used in a public health context. The Innovative Vector Control Consortium (*11,29*) is a public-private partnership that has made great strides in this endeavor. Another strategy that may lessen selection pressure on a single insecticide is the use of larvicides, for which more options are currently available than for adulticides. If larvicides become an important part of the vector control program in the future, resulting data will need to be incorporated into the IRMP.

Discussion

The impact of the policy changes (i.e., to alternate insecticides and cease pyrethroid use) on the reversal of resistance, mosquito abundance, and malaria incidence is currently being monitored, and preliminary results are promising. In some areas of Eastern province, before the switch to Actellic 300CS, *An. funestus* sensu lato mosquitoes showed a very high intensity of resistance to deltamethrin (up to 40% survival at 10 times the diagnostic dose by using CDC intensity assays). Currently, after 2 years of organophosphate spraying, preliminary results indicate a substantial reduction in the intensity of resistance to deltamethrin. Because resistance mechanisms are generally considered biologically costly (*30*), deltamethrin resistance may be decreasing in the absence of any strong pyrethroid selection pressure. However, monitoring is needed to confirm the reversal of resistance and to establish the causative mechanism. The effects of the policy on entomologic and epidemiologic parameters are still being monitored; recently increased efforts include quantifying entomologic indicators of transmission throughout sprayed areas and conducting biannual malaria indicator surveys to track disease trends over time.

A key to the success of Zambia's insecticide resistance management policy development has been the establishment of a multidisciplinary IRMTWG and the expertise of the TAC. These 2 bodies serve as the fulcrum of the entire policy development process by defining objectives, reviewing progress, and actively responding to feedback from policy implementers. This oversight has resulted in effective collaborations among stakeholders and has facilitated accumulation of entomologic data, greater understanding of resistance mechanisms, and establishment of a shared database for insecticide resistance data. However, the success of this policy change relies on continued investment in monitoring and evaluation, industry's development of insecticides with new modes of action, and the building of capacity and infrastructure so that reliance on donor funding and resources can be lessened in the future. Although sentinel sites for entomologic monitoring increased over time on a national scale, adoption of more cost-effective monitoring schemes, such as community-based surveillance (*31*), is needed to ensure sustainability.

Insecticide resistance will contribute to disease reemergence if not managed appropriately (*5*). Although the malaria control community is currently at the forefront of this issue, other vectorborne infections for which control measures rely heavily on the use of insecticides should be proactively mitigating the effects of insecticide resistance on transmission. Other vectorborne infections that are endemic in Zambia (e.g., lymphatic filariasis and dengue) do not have control programs that implement vector control. However, dengue control in other areas relies extensively on the use of insecticides to control immature stages of insects, and resistance to organophosphates and pyrethroids is widespread in the primary vector *Aedes aegypti* mosquitoes (*32*). The control of triatomine bugs that transmit Chagas disease is primarily accomplished through the spraying of residual insecticides in houses (*33*). However, pyrethroid-resistant vector populations are now widespread (*34*). Visceral leishmaniasis, a disease transmitted by phlebotomine sandflies, is also largely controlled by IRS, but various populations have been found to be resistant to the insecticides used in these applications (*35,36*). Resistance monitoring and management must be integrated into all vectorborne disease control programs so that available insecticides can be used judiciously and the efficacy of chemical-based control can be sustained for the long term.

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SYNOPSIS

Rickettsia parkeri Rickettsiosis, Arizona, USA

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Release date: April 13, 2016; Expiration date: April 13, 2017

Learning Objectives

Upon completion of this activity, participants will be able to:

- Assess the clinical presentation of infection with *Rickettsia parkeri*
- Compare the workup and prognosis of infections with *R. parkeri* and *R. rickettsii*
- Evaluate the laboratory evaluation of cases of *R. parkeri* infection

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In the United States, all previously reported cases of *Rickettsia parkeri* rickettsiosis have been linked to transmission by the Gulf Coast tick (*Amblyomma maculatum*). Here we describe 1 confirmed and 1 probable case of *R. parkeri* rickettsiosis acquired in a mountainous region of southern Arizona, well beyond the recognized geographic range of *A. maculatum* ticks. The likely vector for these 2 infections was identified as the *Amblyomma triste* tick, a Neotropical species only recently recognized in the United States. Identification of *R. parkeri rickettsiosis* in southern Arizona demonstrates a need for local ecologic and epidemiologic assessments to better understand geographic distribution

and define public health risk. Education and outreach aimed at persons recreating or working in this region of southern Arizona would improve awareness and promote prevention of tickborne rickettsioses.

Rickettsia parkeri, a tickborne bacterium that causes a febrile, eschar-associated illness throughout many countries of the Western Hemisphere, is transmitted by *Amblyomma* ticks. In the United States, ≈40 cases of *R. parkeri* rickettsiosis have been reported since its recognition in 2004 (*1*). The Gulf Coast tick (*Amblyomma maculatum*) is the principal vector of *R. parkeri* in the United States (*2*), and all previously documented US infections arose within the known geographic range of these ticks (*1*). Confirmed cases of *R. parkeri* rickettsiosis also have been reported from Uruguay and Argentina, where *A. triste* and *A. tigrinum* ticks serve as the principal vector species (*3*–*6*). Recent reviews of tick collection records and archived specimens documented and identified the presence of ticks very closely related to *A. triste* in several regions of the southwestern United States and adjacent regions of Mexico since at least 1942 (*7*,*8*). Here we report 1 confirmed and 1 probable case of *R. parkeri* rickettsiosis, each acquired in southern Arizona after bites from *A. triste* ticks.

Case Histories

Patient 1

Patient 1 was a 49-year-old male resident of Arizona. In July 2014, he was hiking in the Pajarito Mountains of Santa Cruz County, Arizona. This remote and semi-arid region receives a mean annual precipitation of 430 mm and is situated at ≈1,200 m above sea level (Figure 1, panel A). During the hike, the man removed and discarded an adult tick he found attached to his right arm. The tick had been attached for <3 hours. A similar tick found crawling on the patient was photographed on the same day (Figure 1, panel B). An ulcerated lesion appeared at the site of the tick bite ≈5 days later. Ten days after the tick bite, the man had onset of fever with a temperature reaching 38.7°C, which was accompanied by headache, myalgia, and scalp tenderness. On day 11, his physician noted a 1-cm eschar, surrounded by a ring of erythema, lateral to the antecubital fossa of his right arm (Figure 2, panel A). No rash or lymphadenopathy was noted. The patient was prescribed doxycycline (100 mg 2×/d for 10 days), and his temperature returned to normal within 24 hours. However, a sparse maculopapular rash subsequently developed on his back, flank, abdomen, and feet; this rash improved within 4 days. The patient reported no recent out-of-state travel or other tick exposures during the several weeks preceding his illness. A medical entomologist (J.W.M.), expert in *Amblyomma* tick identification and familiar with previous specimens in this genus collected from southern Arizona, reviewed the tick photograph associated with the case and, on the basis of the distinctive dorsal ornamentation of the tick and its geographic origin, presumptively determined the specimen to be an adult male tick of the *A. triste* species. In July 2015, the patient hiked with several other persons in the Pajarito Mountains, ≈5 miles south of where he had sustained a tick bite the preceding year. He and 1 of his hiking companions (patient 2) were bitten by ticks that visually resembled those observed in 2014. The tick that bit patient 1 in 2015 was attached for <8 hours before it was removed. Patient 1 developed a small, erythematous papule with a central depressed scab at the bite site that healed within several days but remained otherwise asymptomatic.

Patient 2

Patient 2 was a 42-year-old female resident of Arizona. While hiking, she discovered a tick attached to her scalp behind her right ear. The tick was attached for <8 hours before it was removed. A small ulcer surrounded by a narrow rim of erythema developed at the bite site. Four days after the tick bite, the patient had onset of fever with a temperature of 37.7°C, myalgia, and fatigue. Two days later, a sparse maculopapular rash appeared on her lower legs

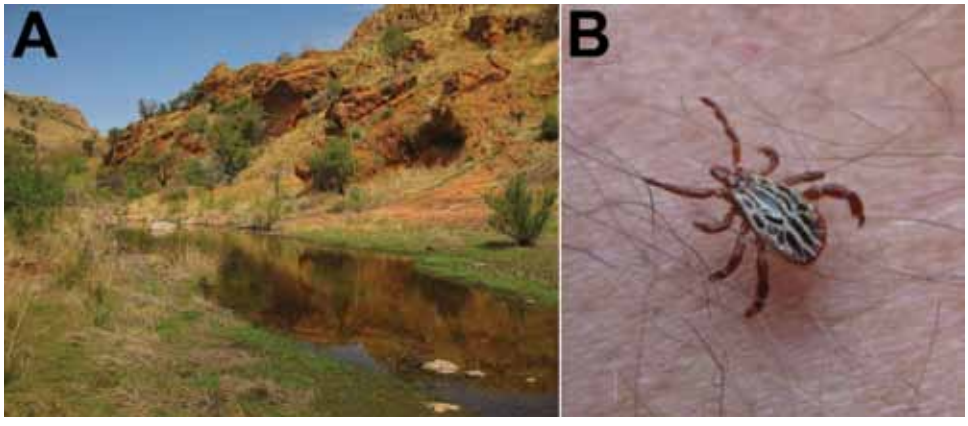

Figure 1. A) Typical habitat in the Pajarito Mountains in Santa Cruz County, Arizona, USA, near the location where patient 1 sustained a bite from a tick that resulted in *Rickettsia parkeri* rickettsiosis in July 2014. B) Male tick identical to the tick that bit patient 1. The distinctive white ornamentation on the scutum and disjunct geographic origin strongly support its presumptive identification as *Amblyomma triste*.

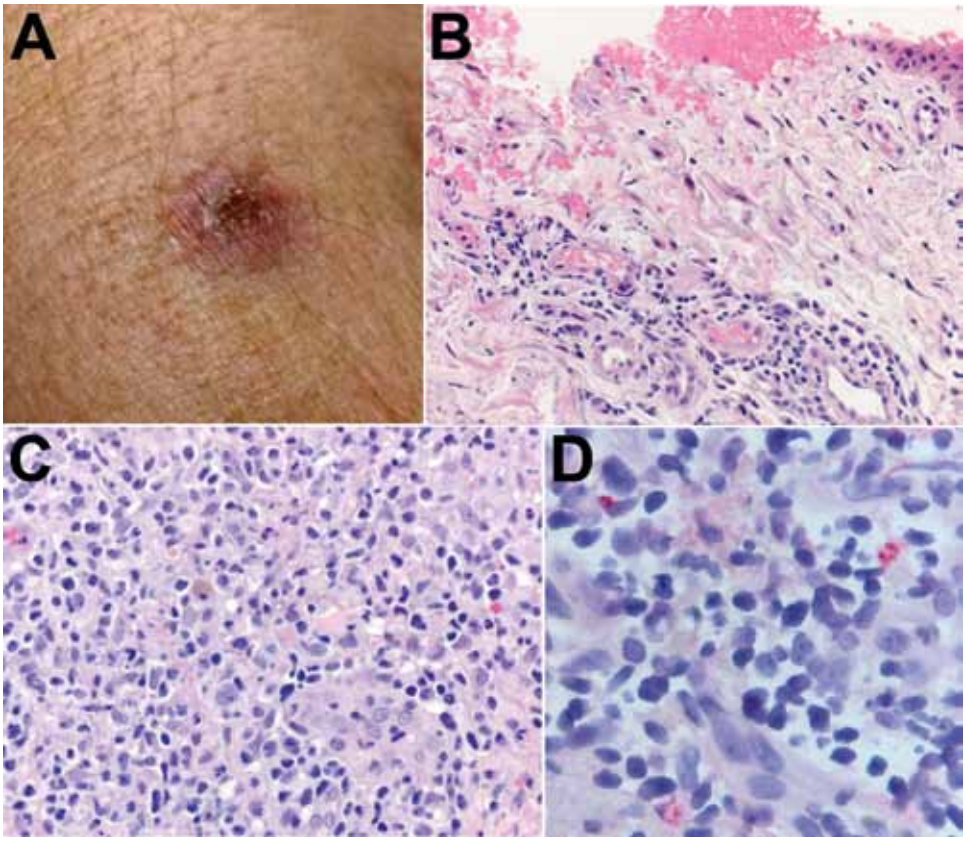

Figure 2. A) Eschar on the right arm of patient 1 at the site of tick bite sustained in Santa Cruz County, Arizona, USA. B) Histological appearance of the eschar biopsy specimen showing ulcerated epidermis with hemorrhage and perivascular lymphohistiocytic inflammatory infiltrates in the superficial dermis. Hematoxylin-eosin staining; original magnification ×50. C) Dense lymphohistiocytic infiltrates around eccrine ducts in the deep dermis of the biopsy specimen. Hematoxylin-eosin staining; original magnification ×100. D) Sparsely distributed intracellular antigens of *Rickettsia parkeri* (red) within the inflammatory infiltrates, detected by immunohistochemistry. Alkaline phosphatase with naphthol-fast red and hematoxylin counterstaining; original magnification ×158.

and arms; this rash lasted for ≈3 days. The patient was prescribed doxycycline (100 mg 2×/d for 10 days) on the first day of fever, and her constitutional symptoms resolved within 48 hours. She did not report any out-of-state or other outdoor exposures during the weeks before her illness.

Materials and Methods

Two tissue biopsy specimens were collected in July 2014 from the eschar of patient 1. DNA was extracted from 1 sample using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) and eluted in a final volume of 200 μL. Extracted DNA was tested in duplicate by using *Rickettsia* genus–specific, *R. rickettsii*–specific, and *R. parkeri*–specific real-time PCR assays (*9*,*10*). Cycle threshold (C_t) values <40 were considered positive. A nested PCR assay was used to amplify a segment of the *ompA* antigen gene by using 3 μL of purified DNA template and 0.8 μmol/L each of primer 190–70 and 190–701 in the primary reaction and 1 μL of the completed primary PCR reaction and 0.8 μmol/L each of primer 190-FN1 and 190-RN1 in the nested reaction (*2*). The amplified DNA fragment was sequenced by using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence alignments were made by using SeqMan Pro in the DNASTAR Lasergene 12 suite (DNASTAR, Inc., Madison, WI, USA) and evaluated with BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi). A second biopsy specimen was fixed in 10% neutral-buffered formalin and embedded in paraffin. Tissue sections cut at 3 μm in thickness were stained with hematoxylin-eosin and tested by using an immunoalkaline phosphatase technique with a polyclonal anti–*R. rickettsii* antiserum, diluted 1:500 (*11*).

Acute- and convalescent-phase serum samples were collected from patient 1 in 2014 and 2015 and from patient 2 in 2015. These specimens were tested for IgG and IgM reactive with antigens of *R. parkeri* and *R. rickettsii* by using an indirect immunofluorescence antibody assay. All specimens were diluted initially at 1:32, and antibody titers were expressed as the reciprocal of the last subsequent dilution that provided specific fluorescence. An antibody titer ≥64 was considered evidence of past exposure to a spotted fever group *Rickettsia* species, and a ≥4-fold change in titer between specimens collected separately was considered evidence of a recent infection or exposure (*11*). Serum samples were processed by using the Zorba IgG Removal Kit (Zeus Scientific, Branchburg, NJ, USA) before evaluating for IgM.

In 2015, the 2 male ticks that had bitten patients 1 and 2, respectively, and an additional 4 female and male specimens found crawling on clothing of the patients and their hiking companions were placed in 70% ethanol. These specimens were sent to the US Department of Agriculture National Veterinary Services Laboratories (Ames, Iowa,

USA) for morphologic identification, and they were subsequently tested at the Centers for Disease Control and Prevention (Atlanta, Georgia, USA) by molecular techniques for evidence of infection with *R. parkeri*. Ticks were minced individually by using sterile scalpel blades. DNA was extracted by using a QIAamp DNA Mini Kit, and each sample was eluted in a final volume of 100 µL. Extracted DNA was evaluated by using a *Rickettsia* genus–specific real-time PCR assay and a nested *ompA* antigen gene PCR assay followed by sequencing, as described previously. An additional collection of 1 male and 1 female *A. triste* tick taken from clothing of the same hikers in Santa Cruz County, Arizona, was retained as a voucher in the parasitology reference collection at the National Veterinary Services Laboratories (accession no. 15-023437).

Results

Real-time PCR of DNA extracted from the eschar of patient 1 yielded positive results when evaluated using the *Rickettsia* genus–specific assay (averaged C_t 36.86, SD 0.74) and the *R. parkeri*–specific assay (C_t 35.51) and yielded a negative result when evaluated using the *R. rickettsii*–specific assay. A 540-bp segment of the *ompA* gene amplified by a nested PCR assay demonstrated complete identity with the corresponding segment of the *ompA* gene of *R. parkeri* strain Portsmouth (GenBank accession no. CP003341.1). Microscopic examination of the formalin-fixed skin biopsy specimen demonstrated ulceration of the epidermis and lymphohistiocytic inflammatory cell infiltrates distributed predominantly around small blood vessels and eccrine glands and ducts in the superficial and deep dermis. Inflamed vessels revealed focally swollen endothelial cells but no fibrin thrombi (Figure 2, panels B and C). Immunohistochemical staining for spotted fever group *Rickettsia* spp. revealed sparse intracellular antigens of *R. parkeri* within macrophages in the inflammatory infiltrates (Figure 2, panel D).

Serum samples from patient 1, collected 6 and 24 days after the onset of his illness in 2014, reacted with *R. parkeri* antigens at IgG titers of 64 and 512, respectively, and with *R. rickettsii* antigens at IgG titers of <32 and 128, respectively. Samples from these 2 collection dates demonstrated IgM titers of 1,024 and 1,024 when these were reacted with *R. parkeri* and *R. rickettsii*, antigens, respectively. Serum samples collected from this patient in 2015, at 12 and 34 days after his second tick bite, reacted with *R. parkeri* antigens at IgG titers of 32 and 128, respectively, and with *R. rickettsii* antigens at IgG titers of 32 and 128, respectively. No IgM reactive with *R. parkeri* or *R. rickettsii* antigens was detected in either sample. Serum specimens collected from patient 2 at 1 and 32 days after the onset of fever reacted with *R. parkeri* antigens at IgG titers of 64 and 64, respectively, and with *R. rickettsii* antigens at IgG titers of <32 and <32, respectively. No IgM reactive with *R. parkeri* or *R. rickettsii* antigens was detected in either sample.

By using the only published taxonomic key that includes *A. triste* among North American *Amblyomma* spp. ticks (*8*) in addition to 2 widely accepted taxonomic keys to Neotropical ticks (*12*,*13*), each of 2 female and 4 male tick specimens collected in 2015 were identified as *A. triste* on the basis of details of scutal ornamentation, leg armature, and festoons. The *Rickettsia* genus–specific real-time PCR assay was positive for 3 male ticks (average C_t 18.36–19.93, SD 0.05–0.09), including the 2 ticks that bit patients 1 and 2. A 540-bp segment of the *ompA* gene was amplified by using nested PCR on each of these same specimens, and sequence analysis demonstrated complete identity with the corresponding segment of the *ompA* gene of *R. parkeri* strain Portsmouth.

Discussion

Before this report, all documented US cases of *R. parkeri* rickettsiosis occurred within the known geographic range of *A. maculatum* ticks, predominantly in coastal states of the Eastern Seaboard and along the Gulf of Mexico (*1*). The patients described in this report were infected with *R. parkeri* in southern Arizona after bites from ticks identified photographically and morphologically as *A. triste*. To our knowledge, established US populations of the Gulf Coast tick do not occur west of the 100th meridian. In contrast, collection records from multiple sources documented historical attachments of *A. triste* ticks to humans in Cochise (1942) and Santa Cruz (1992) Counties in southern Arizona (*7*). Adult *A. triste* tick collections have been reported from these counties during July–September, corresponding with the local monsoon season.

A. triste is an aggressive, human-biting tick species related closely to *A. maculatum* (*14*) and is recognized as a potential vector of *R. parkeri* in Argentina, Brazil, and Uruguay, where rates of rickettsial infection in this tick species range from ≈6% to 20% (*15*–*20*). The distribution of *A. triste* ticks in North America is less well-characterized, with validated collection records from the edges of the Chihuahuan Desert, generally at higher altitudes, and in the Mexican Highlands section of the Basin and Range Province, within the US states of Arizona and Texas (*7*) and the states of Sonora, Durango, and Coahuila in Mexico (*7*,*21*). Our findings indicate that the *A. triste* tick is also a vector of *R. parkeri* in southern Arizona. Although *A. triste* ticks have probably adapted to certain semi-arid environments of the southwest, preliminary observations from this investigation and archival collection records suggest that host-seeking adult *A. triste* ticks are most active during July–September, corresponding to the monsoon season in this region and the period of highest risk for human exposure to *R. parkeri*. *R. rickettsii*, the agent of Rocky Mountain

spotted fever (RMSF), is also endemic to southern Arizona and northern Mexico, where it is transmitted to humans by *Rhipicephalus sanguineus* ticks (*22*,*23*).

The clinical characteristics of the confirmed and probable cases of *R. parkeri* rickettsiosis described in this report are similar to previous descriptions of the disease (*4*–*6*,*11*). Of particular interest is the re-exposure of patient 1 to an *R. parkeri*–infected tick ≈1 year after primary infection with this agent. During his initial infection in 2014, the patient generated substantial titers of IgG and IgM reactive with antigens of *R. parkeri* and *R. rickettsii*. In 2015, after the bite of another infected tick, this patient had a small and rapidly healing lesion at the inoculation site and demonstrated an IgG seroconversion to these same antigens, but did not otherwise become ill and did not mount a measurable IgM response to either antigen. In this context, these data identified an anamnestic antibody response after exposure to an infected tick in 2015 and suggest that some level of protective immunity to *R. parkeri* persisted in patient 1 for at least 1 year after his primary infection.

Future studies should aim to better identify the geographic and host ranges of *A. triste* ticks in the southwestern United States and the frequency with which these ticks are infected with *R. parkeri.* Nonetheless, our data suggest that at least some of the ≈330 cases of RMSF reported from Arizona during the past 10 years (http://www.azdhs.gov/phs/oids/data/stats-archive.htm) might actually represent infections with *R. parkeri*. Because the geographic distribution of *A. triste* ticks also includes several states of northern Mexico, some cases of spotted fever group rickettsiosis in this region might be attributable to infections with *R. parkeri*. Commonly used serologic tests do not distinguish between these clinically similar tickborne diseases, and molecular assays are necessary to provide an etiologic diagnosis (*11*). RMSF is a life-threatening infection that was associated with a 7% case-fatality rate in Arizona during 2002–2011 (*24*) and a 20% case-fatality rate among patients <19 years of age in Sonora, Mexico, during 2004–2013 (*25*). By comparison, no deaths have been attributed to *R. parkeri* rickettsiosis (*1*,*4*,*6*,*11*). Although RMSF and *R. parkeri* rickettsiosis both respond rapidly to therapy with doxycycline, species-specific diagnoses are crucial to accurately define the epidemiologies of the individual diseases in regions where both pathogens might be endemic.

Identification of *R. parkeri* rickettsiosis in southern Arizona demonstrates a need for local ecologic and epidemiologic assessments to better understand geographic distribution and define public health risk. Education and outreach aimed at persons recreating or working in this region of southern Arizona would improve awareness and promote prevention of tickborne rickettsioses.

Ms. Herrick is an infectious disease epidemiologist at the Arizona Department of Health Services. She is interested in the public health response to and epidemiology of communicable diseases.

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Plasmodium falciparum K76T *pfcrt* Gene Mutations and Parasite Population Structure, Haiti, 2006–2009

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Hispaniola is the only Caribbean island to which *Plasmodium falciparum* malaria remains endemic. Resistance to the antimalarial drug chloroquine has rarely been reported in Haiti, which is located on Hispaniola, but the K76T *pfcrt* (*P. falciparum* chloroquine resistance transporter) gene mutation that confers chloroquine resistance has been detected intermittently. We analyzed 901 patient samples collected during 2006–2009 and found 2 samples showed possible mixed parasite infections of genetically chloroquine-resistant and -sensitive parasites. Direct sequencing of the *pfcrt* resistance locus and single-nucleotide polymorphism barcoding did not definitively identify a resistant population, suggesting that sustained propagation of chloroquine-resistant parasites was not occurring in Haiti during the study period. Comparison of parasites from Haiti with those from Colombia, Panama, and Venezuela reveals a geographically distinct population with highly related parasites. Our findings indicate low genetic diversity in the parasite population and low levels of chloroquine resistance in Haiti, raising the possibility that reported cases may be of exogenous origin.

Several decades since malaria has been eradicated from most Caribbean islands, the vectorborne parasitic disease continues to cause sporadic outbreaks in the region and remains endemic only to the island of Hispaniola (*1*), which is the location of the Dominican Republic and Haiti. Evidence suggests that, as in Central America north of Panama, the circulating *Plasmodium falciparum* malaria parasite, which is the dominant malarial species in Haiti and causes illness associated with the highest number of deaths worldwide (http://www.who.int/mediacentre/factsheets/fs094/en/), has remained chloroquine sensitive. The presence of chloroquine-resistant (CQR) parasites in Haiti could have a notable effect on the populace, as well as complicate ongoing efforts of disease control and the ultimate goal of disease eradication. In addition to social and ethical considerations, the eradication of malaria could ultimately aid in alleviating poverty, a particularly critical issue in Haiti, which has the lowest per capita income in the Western Hemisphere (*2*). The known negative effects of malaria on economic growth and human capital development would be amplified because of cost increases for malaria treatment if drug-resistant parasites were to become endemic.

Considering the potential ramifications of the establishment of endemicity of drug-resistant malaria parasites, several surveys have assessed the presence of CQR parasites in Haiti. These studies have predominantly relied upon detection of mutations in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene (*3*–*8*) as a proxy for possible drug resistance. Although the presence of a single point mutation does not prove clinical resistance, the substitution at position 76 from lysine (K76) to threonine (T76) is a useful surrogate marker. This technique uses the nested PCR amplification of *pfcrt* gene sequences, which may be easily detected by using DNA extracted from whole blood spotted on filter paper. The filter papers are dried, then stored with desiccant at collection points until conditions for transportation are favorable. Conventionally, the mutation has been detected by mutation-specific restriction–endonuclease digestion of the *pfcrt*-nested PCR fragments with *Apo*I; the gene sequence containing the T76 threonine substitution

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associated with chloroquine resistance is resistant to digestion, and that containing the wild-type lysine (K76) associated with drug sensitivity reveals 99-bp and 46-bp fragments on agarose gel electrophoresis (*3*–*8*). Because DNA sequencing has become more widely available and routinely practical, direct sequencing of the nested *pfcrt* PCR product for the presence of the mutation has been used (*5*–*8*).

Surveys in Haiti have intermittently detected parasites harboring the CQR haplotype of the *pfcrt* gene (*6*,*9*). In addition, the presence of drug-resistant parasites as assessed by using in vitro culture has been reported (*9*). Clinical chloroquine treatment failures have not been reported, however, and sustained transmission of drug-resistant parasites is not believed to have occurred.

To clarify the genetic context of malaria parasites from Haiti, we used molecular barcode approaches to assess the genetic diversity of this parasite population in the context of geographically distinct neighboring parasite populations. The molecular barcode was used to assess the multiplicity of infection and parasite relatedness. This approach has been used to assess relative parasite transmission intensity (*10,11*) and has the potential to track parasites to better understand their relatedness and sources, including in outbreak investigations (*12*). In addition, we genetically characterized a subset of the parasite population in Haiti sampled by single-nucleotide polymorphism (SNP) molecular barcoding to determine their relatedness to other parasite populations in the region.

We report the findings of our surveillance for parasites harboring *pfcrt* CQR haplotypes in patients with suspected malaria at 9 medical sites across Haiti during the 4 years preceding a major earthquake in January 2010. The earthquake destroyed health infrastructure in the country, including the Ministry of Health and Population, killed >220,000 people, and left >1.5 million homeless. In the aftermath of this catastrophe, major efforts were deployed to establish enhanced surveillance systems to detect and prevent the transmission of disease in the affected population (*13*,*14*).

Materials and Methods

Sample Collection and DNA Processing

Surveillance for CQR *P. falciparum* in Haiti was continuous during 2006–2009 by the Haitian Group for the Study of Kaposi's Sarcoma and Opportunistic Infections (GHESKIO) at 9 healthcare centers in the municipalities of Jeremie, Jacmel, Les Cayes, Miragoane, Cap-Haitien, Deschappelles, Port-au-Prince, Fort-Liberte, and Port-de-Paix. Filter paper (Whatman 3MM, Whatman Corporation, Florham Park, NJ, USA) was cut into 2 cm × 1.5 cm strips. We cut 4 teeth, each with a width of ≈5 mm, in the lower half of the filter paper. Whole blood was obtained by finger prick for absorption on the filter paper teeth (≈50 mL per tooth), and smears from patients with fever and suspected malaria were prepared for parasite detection. The filter papers were dried and stored at room temperature in sealed bags with desiccant. We periodically transferred samples and blood smears to GHESKIO in Port-au-Prince. All blood smears were reviewed at GHESKIO for the presence of parasites. We extracted DNA from the individual filter paper teeth of samples from patients who had parasite-positive smears either by methanol extraction or by using the QIAamp DNA Blood Mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions.

Molecular Analysis of *pfcrt* Gene Sequences

Initially, we analyzed samples for chloroquine resistance mutations by nested PCR amplification of the *pfcrt* gene, then mutation-specific restriction–endonuclease digestion with *Apo*I as previously described (*3*). Positive and negative controls were included in each round of PCR testing: CQR line Dd2, CQS line 3D7, and water alone. Products were resolved and visualized on a 2% agarose gel. Subsequently, we changed the screening method to the direct sequencing of the nested PCR products, either at the Genomics Core of the Cornell University Life Sciences Core Laboratories Core Facility (http://www.biotech.cornell.edu/node/556) or at Macrogen (Rockville, MD, USA). We cloned PCR products using the TOPO TA Cloning Kit (Life Technologies, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Molecular Barcoding

After extraction, molecular barcode data were obtained for a subset of the samples as described previously (*10*). We applied this approach to 72 samples that had sufficient remaining material for processing and included the 2 samples that initially were identified as possibly polygenomic. In brief, 0.3 ng of extracted template material were used in 5 μL total reaction volumes containing TaqMan Universal PCR Master Mix (2×), no AmpErase UNG (Applied Biosystems, Foster City, CA, USA), and 40× TaqMan MGB assays (Applied Biosystems) that were run on a 7900HT real-time system (Applied Biosystems). The samples were genotyped after PCR amplification based on their end-point fluorescence signals (FAM or VIC). Samples that showed >1 mixed-base SNP call or had >5 missing calls in the 24-SNP molecular barcode were removed from analysis.

Data Analysis

We sorted samples for the presence of identical barcodes. Molecular barcodes that shared ≥96% of their positions were defined as highly genetically related. For comparison to other populations in the general geographic region, we performed spatial Principal Component Analysis (sPCA)

Table 1. Evaluation of *Plasmodium falciparum* samples for chloroquine resistance by restriction-endonuclease digestion and sequencing, Haiti, 2006–2009*

Source	No. samples	No. *pfcrt* K76T *Apo*1 sensitive	No. *pfcrt* K76T *Apo*1 combined	No. *pfcrt* K76T sequenced CVMNK	No. *pfcrt* K76T sequenced CVMNK/CVIET
Jeremie	308	73	1†	234	1†
Jacmel	36	14	0	22	0
Les Cayes	316	40	1‡	275	1‡
Miragoane	15	7	0	8	0
Cap-Haitien	4	2	0	2	0
Deschappelles	199	20	0	179	0
Fort-Liberte	13	ND	NA	13	0
Port-de-Paix	3	ND	NA	3	0
Port-au-Prince	7	ND	NA	7	0
Total	901	156	2	743	2

*Sequenced includes sequencing of the nested PCR product for *pfcrt* haplotypes where wild-type haplotype = CVMNK, and mutant haplotype = CVIET; ND, not determined; NA, not applicable; *pfcrt*, *P. falciparum* chloroquine resistance transporter.
†Same sample from Jeremie.
‡Same sample from Les Cayes; pfcrt K76T *Apo*I includes endonuclease digestion and gel electrophoresis. Sensitive: K76; resistant: 76T; *pfcrt* K76T.

using the adegenet function within the PopGenReport package version 2.0 in R version 3.02 (*15*) to analyze monogenomic parasite barcodes from Panama (n = 37) (*12*), Colombia (n = 7) (*12*), and Venezuela (n = 31) (V. Udhayakumar, pers. comm.). These samples were collected during outbreaks in those countries during 2003–2008, 2011–2012, and 2003–2004, respectively.

Results

We analyzed 901 blood samples for the presence of *pfcrt* mutations associated with chloroquine drug resistance (Table 1; Figure 1). Of those, 899 samples were analyzed either by restriction digest or sequencing. Of 158 samples analyzed by *pfcrt* PCR product mutation-specific restriction–endonuclease digestion, 156 showed 99-bp and 46-bp fragments characteristic of the wild-type (chloroquine sensitive) *pfcrt* gene; however, 2 samples repeatedly revealed a fragment resistant to *Apo*I digestion. These samples, 1 each from the cities of Jeremie and Les Cayes, were further analyzed for the possible presence of both chloroquine-sensitive and -resistant parasites. Direct sequencing of the PCR products revealed the potential presence of both alleles. We attempted subcloning of PCR products for these potentially mixed samples to isolate individual PCR products for sequencing, but were not successful. SNP-based molecular barcoding (10) revealed the strain from Jeremie to be a unique isolate (Table 2), but results of the analysis for the isolate from Les Cayes could not be interpreted on 2 occasions because of poor amplification. No CQR *pfcrt* mutations were detected among the remaining 743 samples analyzed solely by direct sequencing. The observation that essentially all samples were confirmed to lack the CQR haplotype for *pfcrt* by molecular approaches supports the clinical observation that chloroquine remains highly effective in Haiti.

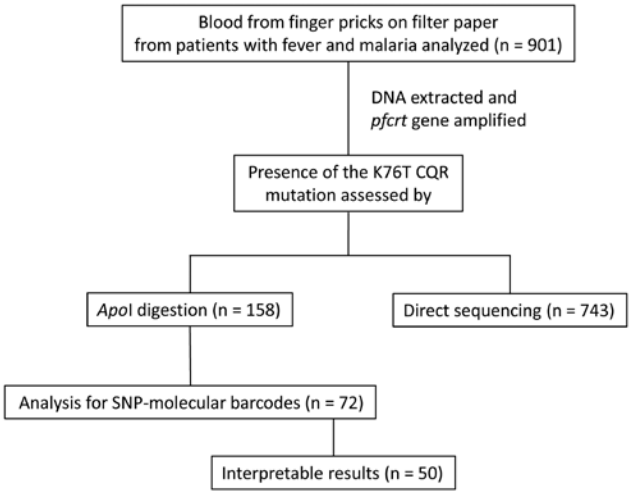

Figure 1. Flowchart of blood specimen processing and analysis for study of presence of *Plasmodium falciparum* K76T *pfcrt* gene mutations, Haiti. Each sample was analyzed for the presence of *pfcrt* mutations associated with chloroquine drug resistance by only one method (restriction digest or sequencing) with the exception of 2 samples. *pfcrt*, *P. falciparum* chloroquine resistance transporter; CQR, chloroquine-resistant; SNP, single-nucleotide polymorphism.

To better understand the genetic relatedness of these parasites endemic to Haiti and discover whether they are related to or different from neighboring parasite populations, we genotyped a subset of samples with sufficient remaining material using an SNP-based molecular barcoding approach. We used this genotyping method on 72 samples collected in 2006 and 2007; 50 (69%) of these yielded interpretable barcodes; 42 (84%) harbored monogenomic and 8 (16%) polygenomic infections (Tables 2, 3). Further comparative analysis of the 42 monogenomic barcodes revealed a high degree of similarity between the parasite genomes (Figures 2,3). Of the 42 parasites, 15 (36%) had identical or nearly identical molecular barcodes, sharing 23 of 24 positions (96% barcode identity) (Figure 3). Furthermore, some of these nearly genetically identical parasites persisted across transmission seasons from 2006 to 2007 (Figure 3; Table 2). Identical parasite barcodes were identified in Deschappelles in 2006

Table 2. Monogenic barcoding data of *Plasmodium falciparum* samples determined to assess genetic relatedness, Haiti, 2006 and 2007

Sample	Location	Year	Molecular barcode*	Type	Category
15	Deschappelles	2006	TACTGCGGATTACCACCAAACTTG	A	Identical
35	Les Cayes	2007	TACTGCGGATTACCACCAAACTTG		Identical
22	Jeremie	2007	CACTCCGGATCXTCAACAAACTTG	B	Identical
23	Jeremie	2007	CACTCCGGATCXTCAACAAACTTG		Identical
31	Les Cayes	2007	TACTGGGGACTACACCCTAGCTTG	C	Identical
33	Les Cayes	2007	TACTGGGGACTACACCCTAGCTTG		Identical
38	Les Cayes	2007	TACTCCGGATCGTCCACAAGCTTG	D	Identical
44	Les Cayes	2007	TACTCCGGATCGTCCACAAGCTTG		Identical
27	Les Cayes	2007	TACTCCGGATTXTCCACAAGCTTG		Related
6	Miragoane	2006	TACTCCAGACTXCCAACTCGATTG	E	Related
36	Les Cayes	2007	TACTCCAGACTACCAACTCGATTG		Related
11	Deschappelles	2006	TATCCCGGATTACCACCTAACCTG	F	Related
16	Deschappelles	2007	TATCCCGGATTAXCCCCTAACCTG		Related
30	Les Cayes	2007	TACTGCGGATXACACACTAGCCTG	G	Related
43	Les Cayes	2007	TACTGCGGATTXCACACAAGCCTX		Related
5	Miragoane	2006	XACTCCGGACTXCAACCAAACTTG	U	Unique
7	Miragoane	2006	TACTCCAGACTACCAACTCGACGG	U	Unique
8	Deschappelles	2006	TATTGCGGATTXCCCCCTAACTTG	U	Unique
9	Deschappelles	2006	CACTCCGGATTACACACAAACCTG	U	Unique
10	Deschappelles	2006	TATCCCGGATCATACCCTAACTTG	U	Unique
12	Deschappelles	2006	TACTGCAGATXXCACACTAAACTG	U	Unique
13	Deschappelles	2006	TATTGCAGATCGTCCCCTAGATTG	U	Unique
14	Deschappelles	2006	TACTCCAGATTGTCCCCTAGCTTG	U	Unique
17	Deschappelles	2007	TACTGCGGATCATAACCAAGCTTG	U	Unique
18	Jacmel	2006	TAXTCCGGATTGTCACCAAGCTTG	U	Unique
19	Jacmel	2007	TACTGCGGATCATAAACAAACTTG	U	Unique
20†	Jeremie	2007	TACXCXAGATTXTCXXTACACTTG	U	Unique
21	Jeremie	2007	TATTGCGGATTACACCCTAGCCTG	U	Unique
24	Cap-Haitien	2006	TXCXCCGGAXTXCCCXCAAGXTTG	U	Unique
25	Les Cayes	2006	TACTGCAGATCGTACCTTAGCCTG	U	Unique
26	Les Cayes	2006	TACCCCGGACCGCAACCTAAATTG	U	Unique
28	Les Cayes	2007	TATTCCAGATCGTCCCTTAGACTG	U	Unique
29	Les Cayes	2007	TAXTGCGGATTGTCACCTAACTTG	U	Unique
32	Les Cayes	2007	TACCCCGGATTXCACCCAAACTTG	U	Unique
34	Les Cayes	2007	TACTCCGGACCGCACCCTAAATTG	U	Unique
37	Les Cayes	2007	TACTCCGGACTACCCCCTAACTTG	U	Unique
39	Les Cayes	2007	TATCGCAGATTACCAATAAGCCTG	U	Unique
41	Les Cayes	2007	TATTCCGGATXACCCACTAGCTTG	U	Unique
42	Les Cayes	2007	TATTCGGGATTATCCACTAGATTG	U	Unique
45	Les Cayes	2007	TACXGXAGATTXTCCCCACGCTTG	U	Unique
46	Les Cayes	2007	TACTGCGGACCGXCCCCTAACCTG	U	Unique
47	Les Cayes	2007	TACTGCAGACTXCCCCCACGCTTG	U	Unique

*X indicates missing data, where neither the major or minor allele could be detected.
†Sample 20 from Jeremie revealed the possible presence of both alleles by mutation-specific restriction–endonuclease digestion and DNA sequencing.

and Les Cayes in 2007; highly related parasites were also identified in Miragoane in 2006 and Les Cayes in 2007 and in Deschappelles in 2006 and 2007. Additional comparative analysis of the barcodes from Haiti with those from Panama, Colombia, and Venezuela by using sPCA suggest that Haitian parasites are distinct from these other parasite populations in the region (Figure 4).

Although these samples represent a small subset of the malarial parasite population of Haiti, these genetic analyses show highly related parasites within Haiti that are distinct from geographically neighboring parasite populations. Furthermore, detection of parasites with identical barcode genotypes in 2006 and 2007 suggests that parasite populations may persist from one transmission season to another, which implies the need for intervention strategies such as targeting parasite reservoirs that may harbor such persisting parasites from one transmission season to the next.

Discussion

Malaria remains a major cause of illness and death worldwide. With the exception of Hispaniola, the Caribbean islands are free of sustained *P. falciparum* parasite transmission and report only occasional outbreaks. These outbreaks are likely caused by the importation of parasites by infected persons, leading to local transmission by competent mosquito vectors. However, malaria remains endemic to Haiti and the Dominican Republic on the island of Hispaniola. Consistent with worldwide efforts, the control and eradication of malaria in Hispaniola is deemed warranted and feasible. However, sporadic reports of CQR parasites raise concerns that these efforts may prove more costly and difficult to accomplish than envisioned.

We report on the analysis of 901 samples from 9 sites throughout Haiti collected during the 4 years preceding the magnitude 7.0 earthquake that occurred in January

Table 3. Polygenomic barcodes of *Plasmodium falciparum* samples determined to assess genetic relatedness, Haiti, 2006–2007

Sample	Location	Year	Molecular barcode*
1	Cap-Haitien	2006	TACTGCGGATTNNCCCNAAGCTTG
2	Deschappelles	2006	TATCCCGGATCANACCCTANCNTG
3	Deschappelles	2007	TATCCCNGATCATACCCTAACCTG
4	Deschappelles	2007	TACTNCNGATTNNNCNCAANCNTG
5	Jeremie	2007	TACTNCNGATCGTNACNTANNTTG
6	Les Cayes	2006	CACXGXGXATCXTAAXCTAGNCTG
7	Les Cayes	2007	TATXCXGGATNXCCCCCACGCNTG
8	Les Cayes	2007	TACTCCGGATCGTCCANAAGCTTG

*X indicates missing data, where neither the major or minor allele could be detected; N indicates both alleles detected.

of 2010. These surveillance efforts were quickly reestablished in the wake of the disaster in order to monitor and prevent the transmission of malaria among vulnerable groups (*15,16*). Despite prior reports of the presence of parasites in Haiti harboring the *pfcrt* drug resistance mutation, we found that most of these parasites contained the chloroquine-sensitive *pfcrt* allele. Only 2 samples from southern Haiti contained parasites that possibly harbored a mixed population of parasites, including those containing the allele indicative of drug resistance. An extensive evaluation of these samples from patients in Les Cayes and Jeremie, in which both sensitive and resistant alleles were detected by mutation-specific restriction–endonuclease digestion of nested PCR *pfcrt* gene products, was inconclusive. Direct sequencing of the nested *pfcrt* PCR products yielded variable results but also suggested the presence of resistant and sensitive alleles. Cloning to permit sequencing of unique isolates was unsuccessful, as was molecular barcoding of the sample from Les Cayes. Molecular barcoding of the sample from Jeremie, however, revealed a single strain. These findings might be consistent with the presence of parasite populations subclinically harboring the resistant allele. The presence of such low-density infections may be difficult to detect and characterize; therefore, we cannot conclusively rule out the presence of resistant alleles in these 2 samples (*16*). Nevertheless, considering that only 2 of 901 samples could not be confirmed as harboring only a chloroquine-sensitive haplotype for *pfcrt*, our findings indicate that nearly all parasites tested were chloroquine sensitive. We therefore conclude that chloroquine resistance was not likely to be present in Haiti during the analyzed period and that chloroquine remains clinically useful in Haiti.

The observation that increasing use of chloroquine after the earthquake in 2010 did not select for or increase the prevalence of these mutations among the parasite

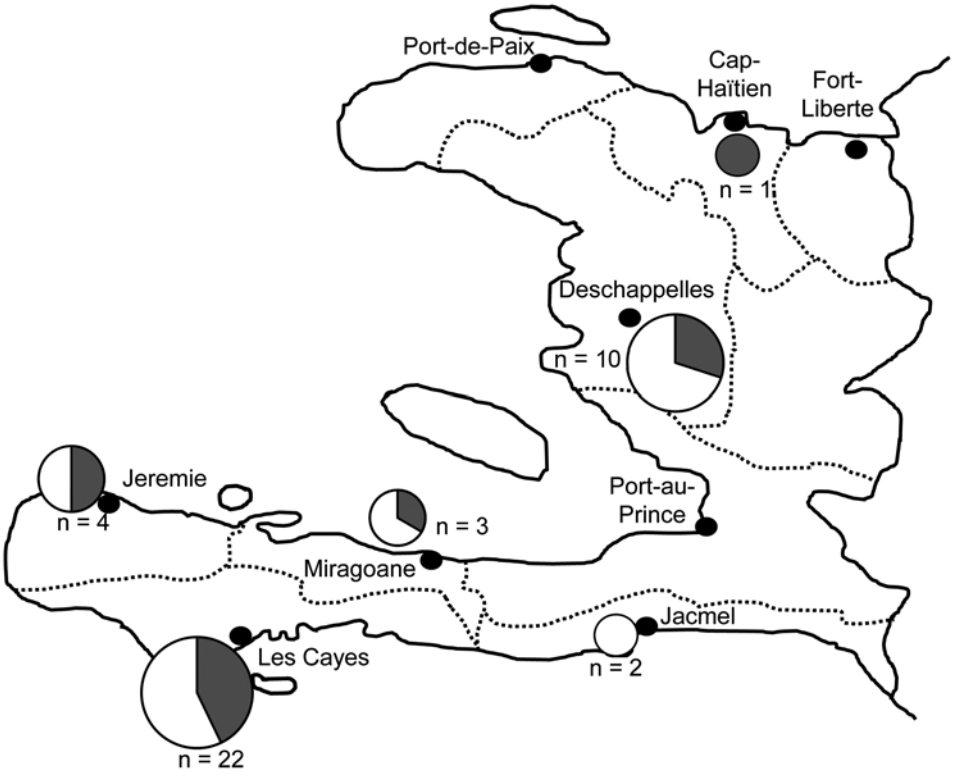

Figure 2. *Plasmodium falciparum* parasite molecular barcode relatedness by site, Haiti. A total of 42 monogenomic samples were obtained from 6 sites (black dots). Circle sizes represent the number of samples from each site. Highly related samples, with either 24/24 or 23/24 identical single-nucleotide polymorphism positions (≥96% barcode identity) are shown in gray circle sections; less related samples (<96% barcode identity) are shown in white circle sections.

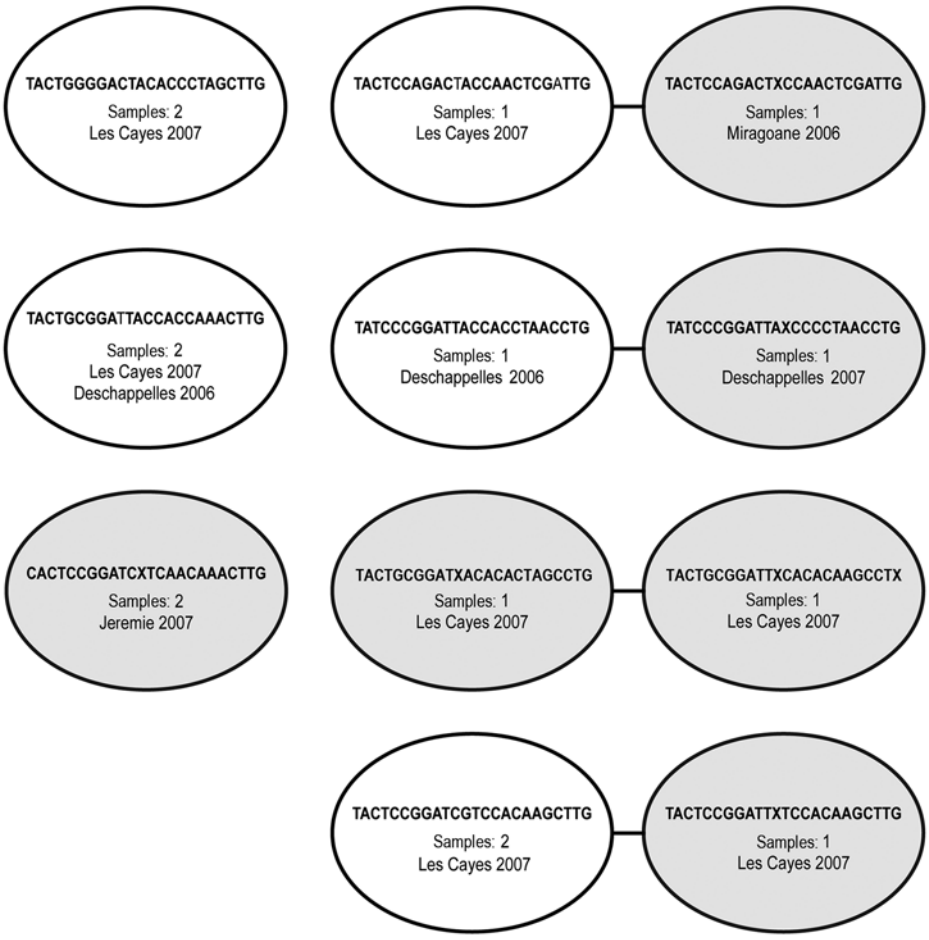

Figure 3. Visualization of 15 identical (same single-nucleotide polymorphism positions call at 24 of 24 positions) and nearly identical (23 of 24 identical positions) molecular barcodes from 42 monogenomic samples from patients in Haiti, 2006 and 2007. Each node (oval) represents an individual barcode. Samples with identical barcodes are included in the same nodes, and related barcodes (1 single-nucleotide polymorphism positions difference) are connected by lines. Gray nodes indicate that there is some point of ambiguity between barcodes, defined as either both alleles detected (N) or no data (X) at a specific position, indicating <100% confidence of a complete match between barcodes.

population as described by Morton et al. (*14*) is consistent with the lack of resistant forms of *pfcrt* among the population sampled in 2006 and 2009 in Haiti. Although a 2011–2012 study conducted by Okech et al. raises concerns regarding drug efficacy related to the persistence of parasites in some patients treated with chloroquine, the resistance mutation was not detected (*17*). A prior report indicated a high percentage of *pfcrt* mutant alleles (5/79, 6%) in *P. falciparum*–positive blood samples in the Artibonite Valley of Haiti in 2006 and 2007 (*6*). Our surveillance included 199 samples from this region but did not show resistant alleles, indicating the transmission in this region may not have been sustained under chloroquine pressure. However, possible resistance cannot be definitively excluded.

We used the molecular barcode data for parasite population genetic analysis; results suggest that the *P. falciparum* parasite population in Haiti is highly related genetically. Of the 42 samples analyzed, 15 (36%) shared ≥23 of the 24 SNPs of the molecular barcode (96% identical barcode). We have shown in a previous study comparing relationships of the molecular barcode and whole-genome sequencing data that molecular barcodes that share ≥75% of their positions are highly genetically related (*11*), indicating that samples described here as identical at 23 or 24 markers in the molecular barcode are genetically or nearly genetically identical.

We increased the threshold to 96% in this study to increase our confidence of identifying truly related parasite types. Furthermore, specific parasite types persist from one year to the next among these highly related parasites, suggesting transmission of single clones without recombination and likely limited introduction of new parasite types through travel or migration. These observations of highly related and clonal parasites that endure across years are consistent with decreasing transmission and potential inbreeding among the parasite population in Haiti. They are also consistent with the population structure analysis of Morton et al. (*14*).

The PCA analysis reveals that the *P. falciparum* parasite population in Haiti is generally separate from and independent of parasite populations from Panama, Colombia, and Venezuela. However, 1 parasite from Colombia clusters with the parasite population in Haiti. This observation is consistent with the possible transfer of parasites

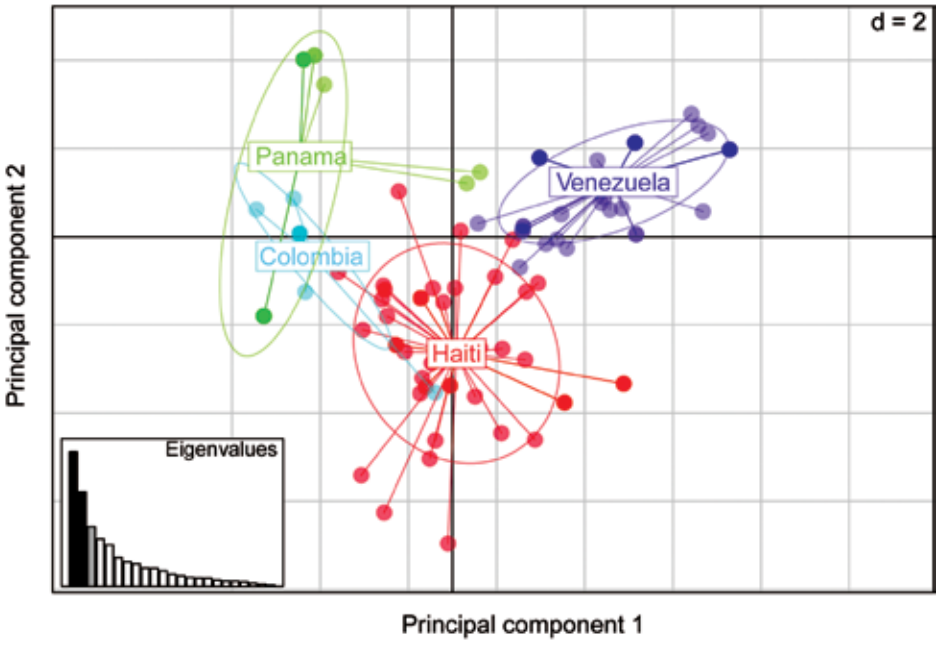

Figure 4. Spatial principal components analysis (sPCA), performed in 2 dimensions (d = 2) comparing malarial parasite population structures based on monogenomic single-nucleotide polymorphism barcodes from Haiti (n = 42), Colombia (n = 7), Panama (n = 37), and Venezuela (n = 31). The x-axis represents the eigenvector associated with the first principal component, which differentiates between populations; the y axis represents the second principal component, which differentiates between samples within the same populations. Inset graph depicts the amount of variability described by the principal components: x-axis indicates individual principal components, y-axis their individual contribution to the observed variance. Black bars, displayed eigenvectors; gray bars, retained principal components; white bars, nonretained principal components.

between this site and Haiti. However, these observations are based on a relatively limited number of samples; although we cannot rule out potential sampling bias, these findings are noteworthy.

After a temporary lull in control efforts related to *P. falciparum* malaria after the 2010 earthquake, attempts at control with an ultimate goal of eradication have resumed (*18*,*19*). Characterization of baseline populations before catastrophic events can support efforts to identify and predict the effects on parasite populations in response to relaxation of control efforts and changed environments. In Haiti, identification of specific parasite genetic types may suggest importation of parasites related to humanitarian efforts or population migration; in addition, the identification of hot spots of transmission of specific parasite types can direct precise application of control efforts, particularly when resources are limited. Our equivocal detection of only 2 patients potentially harboring low-level populations of chloroquine resistance alleles despite screening >900 samples collected during 4 years supports the contention that the sustained propagation of CQR parasites was not occurring in Haiti during the study period; the possibility is raised by Morton et al. that reported cases are of exogenous origin (*14*). The finding of low genetic diversity in the *P. falciparum* population in Haiti is also consistent with that of Morton et al. and others (*7*,*14*,*20*). Although encouraging, these findings support the need for continued surveillance during eradication efforts as well as additional studies to better understand the malarial parasite population structure in Haiti.

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Dr. Charles is a medical epidemiologist at Weill Medical College of Cornell University, New York, who focuses on building capacity in developing countries through training, research, and the transfer of simple and robust diagnostic technology. His research interests include tuberculosis, HIV/AIDS, and emerging infectious diseases.

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Outbreak of Middle East Respiratory Syndrome at Tertiary Care Hospital, Jeddah, Saudi Arabia, 2014

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During March–May 2014, a Middle East respiratory syndrome (MERS) outbreak occurred in Jeddah, Saudi Arabia, that included many persons who worked or received medical treatment at King Fahd General Hospital. We investigated 78 persons who had laboratory-confirmed MERS during March 2–May 10 and documented contact at this hospital. The 78 persons with MERS comprised 53 patients, 16 healthcare workers, and 9 visitors. Among the 53 patients, the most probable sites of acquisition were the emergency department (22 patients), inpatient areas (17), dialysis unit (11), and outpatient areas (3). Infection control deficiencies included limited separation of suspected MERS patients, patient crowding, and inconsistent use of infection control precautions; aggressive improvements in these deficiencies preceded a decline in cases. MERS coronavirus transmission probably was multifocal, occurring in multiple hospital settings. Continued vigilance and strict application of infection control precautions are necessary to prevent future MERS outbreaks.

Middle East respiratory syndrome (MERS) coronavirus (CoV) is a new group C betacoronavirus first reported in a man hospitalized in Jeddah, Saudi Arabia, in June 2012 (*1*). In retrospect, MERS-CoV was found to have caused a respiratory illness cluster in April 2012 in Zarqa, Jordan (*2*). As of January 4, 2016, a total of 1,625 laboratory-confirmed MERS cases, 586 fatal, have been reported from 26 countries; >85% have been reported from Saudi Arabia (*3*). MERS-CoV infection is characterized by fever, cough, and dyspnea, and severity ranges from mild illness to acute respiratory distress, organ failure, and death; the case-fatality rate is ≈36% (*3*–*7*). Most documented symptomatic infections have resulted from human-to-human transmission (*6*). Transmission among healthcare workers (HCWs), hospital inpatients, dialysis patients, and families has been implicated in previous clusters (*2*–*5*,*8*,*9*). MERS-CoV, like other CoVs, is thought to spread through respiratory secretions; however, the precise ways the virus spreads are not well understood (*10*). The role of contact with surfaces contaminated by respiratory secretions (*5*,*11*,*12*) is unclear. Modeling done during other outbreaks estimated the incubation period to be 5.2–6.8 days (*5*,*13*,*14*).

During March 2014, the Saudi Arabia Ministry of Health (MOH) reported to the World Health Organization an increase in MERS cases in the Jeddah area (*7*). Genetic typing suggested this outbreak was caused by transmission of a single viral subtype (*15*); the reason for the increase in cases was unclear (*3*). Subsequent investigation showed that, among symptomatic patients, 21% were HCWs and 88% of evaluable non-HCWs had exposure to healthcare facilities in the 14 days before illness onset (*9*). King Fahd General Hospital (KFGH), an 815-bed public hospital, was the primarily affected facility (*15*). However, multiple MERS patients were treated in >1 healthcare facility, and the number of infections attributable to transmission at KFGH was uncertain. MERS-CoV spread at other healthcare facilities has been documented in dialysis units (*5*);

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early reports indicated that the KFGH dialysis unit might be the primary site of infection transmission.

Risk factors for MERS-CoV transmission at KFGH, and the efficacy of implemented control measures, were uncertain. We investigated the cluster of MERS illnesses at KFGH to determine the number of cases acquired at KFGH, identify hospital areas where transmission occurred, and assess the relationship between the implementation of infection control precautions and MERS cases.

Methods

An assessment of the Jeddah outbreak conducted before our investigation at KFGH (*9*) produced a list of all MERS cases in Jeddah diagnosed by reverse transcription PCR (RT-PCR) conducted at the Jeddah MOH regional laboratory or Jeddah-area hospitals. This list included patient demographics, clinical data, onset date, and exposure data (including exposure to healthcare facilities) (*9*) and was used to identify ill persons with exposure to KFGH.

We defined a case as RT-PCR–confirmed MERS-CoV infection in a symptomatic person during March 2–May 10, 2014, who was exposed to KFGH as a HCW, patient, or visitor. We selected this study period to capture all persons with exposure to KFGH in the 14 days before illness onset. We categorized cases as potentially KFGH-acquired if affected persons had known KFGH contact before MERS onset and not KFGH-acquired if their first contact with KFGH followed MERS onset. We used KFGH electronic records to determine admission and discharge dates, inpatient rooms and beds occupied, and demographics for emergency department (ED) patients and inpatients. We assumed an incubation period of 2–14 days (*5*) and that patients were potentially infectious for 14 days after the onset date. To track the source of infection in individual patients, we looked for instances where an infectious inpatient was in the same room on the same date as a susceptible patient during the 2–14 days before MERS onset in the susceptible patient. We interviewed administrative, infection control, and clinical personnel at KFGH to review infection control recommendations and conducted a facility walk-through to evaluate infection control measures.

Retrospective Cohort Study

To determine setting-specific MERS acquisition rates at KFGH, we conducted a retrospective cohort study among KFGH patients treated in the ED, inpatient areas, or dialysis unit. The cohort comprised all patients who did not have symptomatic MERS when first treated in 1 of these 3 settings. For ED and inpatient areas, we counted patient-days from the first date of treatment until MERS symptom onset, discharge, or study period end; for the dialysis unit, we estimated patient-days as explained in the Hemodialysis Unit subsection. A cohort study case was defined as onset of RT-PCR–confirmed MERS-CoV infection within 2–14 days of treatment in ≥1 of these 3 settings. We excluded 1 patient who had symptom onset the day after admission because the incubation period would have been <2 days after first exposure to KFGH. For patients treated in >1 of the 3 settings, we determined the most probable setting and week of acquisition based on the assumption that the incubation period followed a log-normal distribution with mean 5.2 days and SD ±1.7 days as described by Assiri et.al. (*5*) (Tables 1, 2). Other published incubation period estimates (mean 5.5 days, SD ±2.5 days [*13*]; and mean 6.8 days, no SD provided [*14*]) were used in sensitivity analyses. We calculated incidence rates as cases per 10,000 patient-days, rate ratios, and mid–p values. To support these calculations, we used maximum-likelihood modeling to provide additional estimates of infection rates

Table 1. Probability of Middle Eastern respiratory syndrome coronavirus incubation periods, King Fahd General Hospital, Jeddah, Saudi Arabia, March 2–May 10, 2014*

Incubation period, d	Day probability	Cumulative probability
1	0.0010	0.0010
2	0.0349	0.0359
3	0.1141	0.1500
4	0.1605	0.3105
5	0.1600	0.4705
6	0.1358	0.6063
7	0.1060	0.7123
8	0.0792	0.7916
9	0.0578	0.8494
10	0.0417	0.8911
11	0.0299	0.9210
12	0.0215	0.9425
13	0.0154	0.9579
14	0.0111	0.9690

*Incubation periods of 1–14 days by using a log-normal distribution with mean 5.2 days and SD ±1.7 days[2].

Table 2. Calculation of most probable setting of Middle East respiratory syndrome coronavirus acquisition for a patient with onset date April 2, 2014, King Fahd General Hospital, Jeddah, Saudi Arabia*

Date	Setting where treated	Incubation period, d	Probability
Mar 24	ED	9†	0.0578‡
Mar 25	ED	8	0.0396§
Mar 25	Inpatient	8	0.0396§
Mar 26	Dialysis	7	0.0530
Mar 26	Inpatient	7	0.0530
Mar 27	Inpatient	6	0.1358
Mar 28	Dialysis	5	0.0800
Mar 29	Inpatient	5	0.0800
Mar 29	Dialysis	4	0.0803
Mar 29	Inpatient	4	0.0803
Mar 30	Inpatient	3	0.1141

*Data are from patient 5 (Figure 1). The total probability is 0.50 for inpatient, 0.21 for the dialysis unit, and 0.10 for ED, so acquisition most probably occurred in the inpatient areas. ED, emergency department.
†Represents the incubation period if date of disease acquisition was March 24 and disease onset was April 2.
‡Represents the probability of an incubation period of 9 days (Table 1).
§Patient was treated in 2 settings on March 25, so probability is half of the probability for each setting.

and 95% CIs (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/5/15-1797-Techapp1.pdf).

Hemodialysis Unit

From dialysis unit sign-in logs, we determined the monthly number of patients dialyzed in each of the unit's 4 sections. Assuming each patient was dialyzed 3 times per week (standard practice), we used a linear regression model to estimate the number of patients dialyzed each day. We obtained dialysis dates and beds used by MERS patients from dialysis unit records; for days when this information was unavailable, we assigned the patients to the section where their dialysis usually occurred.

Statistical Analyses and Ethics Considerations

For statistical analyses, we used Excel (Microsoft Corporation, Redmond, WA, USA), SAS version 9.3 (SAS, Incorporated, Cary, NC, USA), Epi Info 7 (http://wwwn.cdc.gov/epiinfo/), and OpenEpi version 3.03a (http://www.openepi.com). This investigation was reviewed by the Centers for

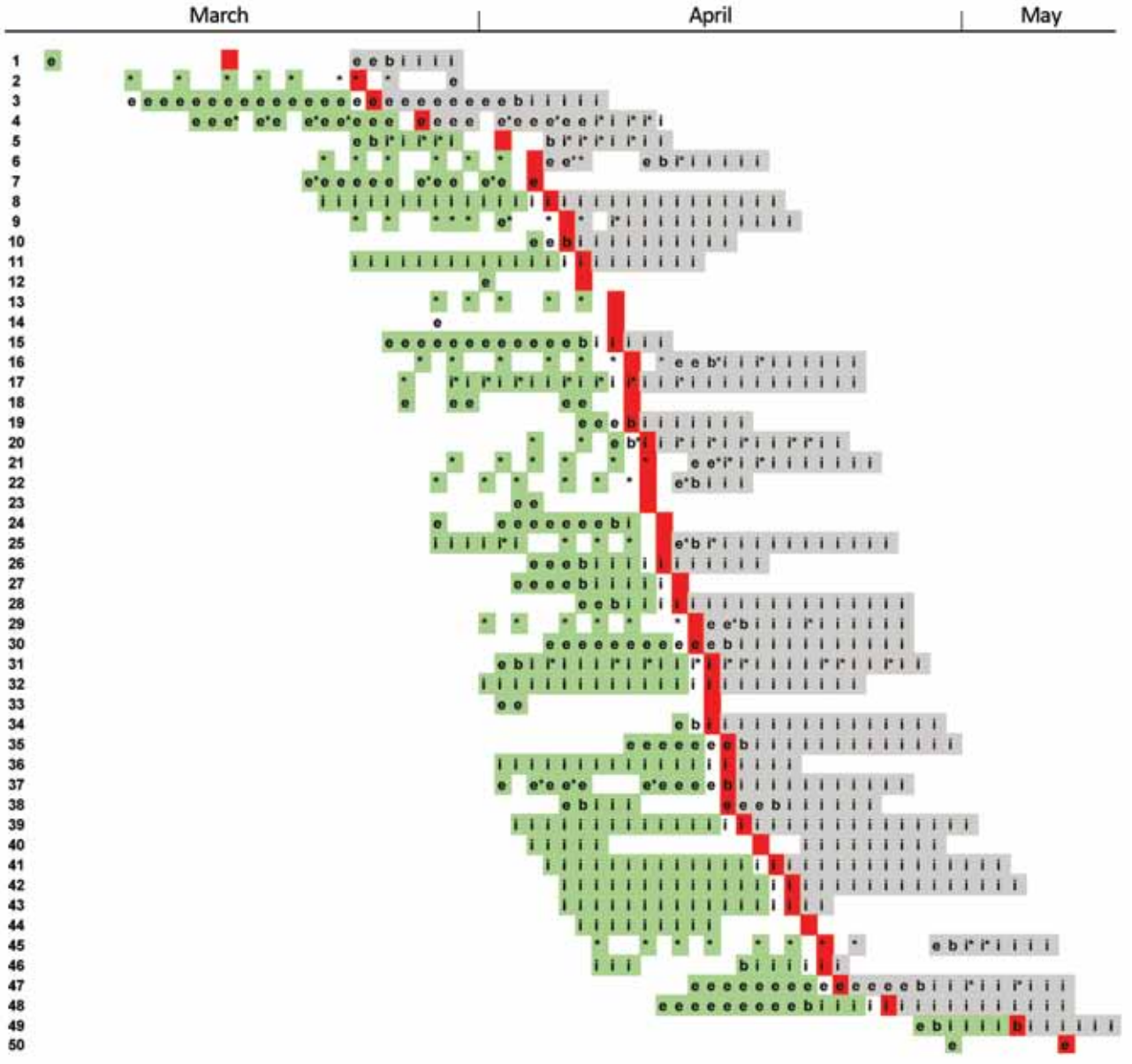

Figure 1. Location of cohort study patients in the 14 days before Middle East respiratory syndrome symptom onset, the day of onset, and 14 days after onset, King Fahd General Hospital, Jeddah, Saudi Arabia, March 2–May 10, 2014. Green indicates the 2–14 days before symptom onset (susceptible period); red indicates the day of onset; gray indicates the 14 days after onset (infectious period). e, emergency department; i, inpatient area; b, emergency department and inpatient areas; * indicates dialysis unit.

Disease Control and Prevention and MOH and was determined to be a nonresearch public health response and not subject to Institutional Board Review.

Results

Patient Characteristics

We identified 130 symptomatic MERS patients who had contact with KFGH. Of these, 52 were not KFGH-acquired and 78 (60%) were potentially KFGH-acquired. The 78 comprised 50 cohort study patients (i.e., treated in ≥1 of the ED, inpatient, or dialysis settings) as well as 16 HCWs, 9 hospital visitors, and 3 outpatients (Table 3). Median age was lower in HCWs and visitors (both 47 years) than in cohort study patients (56 years).

For most of the 78 KFGH-acquired cases, illness onset occurred during April 4–22, 2014 (Figure 1). The median onset date was earlier for the 78 KFGH-acquired cases (April 11) than for the 52 non–KFGH-acquired cases (April 22). Of 16 HCWs with MERS acquired at KFGH, most (10 [63%]) were physicians; the proportion of physicians with MERS infection was much higher (1%) than for other personnel categories (Table 4).

Retrospective Cohort Study

The patient cohort included 2,776 hospital inpatients and 15,218 ED patients; the number of individual dialysis patients could not be determined. Among these, we identified 50 cohort study cases; these 50 patients had a median 6.5 days (interquartile range 5.0–11.0 days) of KFGH treatment during the 2–14 days before symptom onset (when they were susceptible); 41 of the 50 continued to be treated at KFGH (for a median of 12 days) in the 14 days after symptom onset, when they could have infected other patients (Figure 2). When we looked for MERS exposures outside of KFGH among the 50, we found that 1 had an outpatient contact with a known MERS patient and 1 had been admitted to a different hospital for a non-MERS illness. When we looked for documented opportunities for patient-to-patient transmission, we found that only 2 MERS patients were known to have been in the same room as an infectious patient while hospitalized at KFGH. Thirty-two of the cohort study MERS patients were exposed to only 1 setting in the 2–14 days before symptom onset; of these, 14 (44%) were exposed only to the ED, 10 (31%) to inpatient areas, and 8 (25%) to the dialysis unit. When all 50 were categorized according to the probable site of acquisition, acquisition was attributed to the ED for 22 (44%), inpatient areas for 17 (34%), and the dialysis unit for 11 (22%). In sensitivity analyses, varying the mean incubation from 5.2 to 6.8 days, the number of cases probably acquired in the ED varied from 20 to 23 and in the inpatient area from 16 to 19; the number did not change for the dialysis unit.

Overall MERS incidence during the outbreak period was 6.1 cases/10,000 patient-days and was similar (range 5.3–6.5 cases/10,000 patient-days) for the ED, inpatient area, and dialysis unit (Table 5). Incidence was substantially higher during outbreak weeks 5–7 (March 30–April 19; rate 10.3–29.4 cases/10,000 patient-days) than during other weeks (0–4.2/10,000 patient-days) (Figure 3). The incidence rate was higher for non-Saudis than for Saudi nationals (10.1 vs. 4.5 cases/10,000 patient-days; $p = 0.01$) and for older persons (40–59 and ≥60 years, rates 19.1 and 17.4/10,000 patient-days, respectively) than younger persons (0–19 and 20–39 years, rates 2.4 and 5.9/10,000 patient-days, respectively). The illness rate was higher for male than for female persons, but the difference was not statistically significant.

Per 10,000 patient-days, the maximum-likelihood model estimates of incidence rates were 6.4 (95% CI 3.0–9.8) for the ED, 6.2 (95% CI 2.3–10.1) for inpatient areas, and 5.5 (95% CI 1.5–9.5) for the dialysis unit; by using dialysis as the reference category, risk ratios were 1.16 (95% CI 0.45–4.45) for the ED and 1.14 (95% CI 0.36–4.29) for inpatient ares (online Technical Appendix). These values were similar to, and confirmed, results obtained by using the most probable site of acquisition.

Hemodialysis Unit Study

Of the 50 cohort study case-patients, 22 were dialysis patients. Of the 22, the most likely site of acquisition was the dialysis unit for 11, the ED for 7, and inpatient areas for 4. Three of the 11 with dialysis unit acquisition had been dialyzed 2–14 days before their MERS onset in the same dialysis unit section and same shift as an infectious patient. For the 11 with dialysis unit acquisition, the patient-day

Table 3. MERS cases potentially acquired at KFGH, Jeddah, Saudi Arabia, March 2–May 10, 2014*

Category	No. (%) cases	Patient median age, y	No. (%) male patients
All KFGH-acquired cases	78 (100.0)	53	59 (75.6)
Acquisition category			
Cohort study case-patient†	50 (64.1)	56	37 (74.0)
Healthcare worker at KFGH	16 (20.5)	47	12 (75.0)
Visited patient treated at KFGH	9 (11.5)	47	8 (88.9)
Outpatient appointment at KFGH	3 (3.8)	59	2 (66.7)

*Cases were categorized as potentially KFGH-acquired for persons who had KFGH contact before MERS onset. KFGH, King Fahd General Hospital; MERS, Middle East respiratory syndrome.
†Patients with MERS onset 2–14 days after emergency department, inpatient, or dialysis treatment at KFGH. Includes 3 healthcare workers (1 who worked at KFGH and 2 at other hospitals) who were admitted to KFGH inpatient areas or emergency department 2–14 days before MERS onset.

Table 4. Middle East respiratory syndrome coronavirus infections among employees, King Fahd General Hospital, Jeddah, Saudi Arabia, March 2–May 10, 2014

Personnel category	No. employees	No. cases (incidence*), n = 16
Physician	958	10 (1.0)
Nurse	1,260	2 (0.2)
Technician	665	0
Administrator	515	1 (0.2)
Pharmacist	21	0
Maintenance or housekeeping	1,295	1 (0.1)
Other or unknown	†	2

*Per 100 employees.
†Total number of King Fahd General Hospital personnel in this category is unknown.

incidence was not significantly higher in any of the 4 dialysis unit sections than in others (data not shown).

Infection Control Evaluation

Early in the study period, ED patients were treated in 1 of 5 rooms, according to illness acuity. The ED was typically crowded, with patient gurneys nearly touching one another; no system for triaging and isolating patients with respiratory diseases was available, and patients often remained in the ED for multiple days under the care of admitting service physicians. During epidemic week 6 (April 6–12), KFGH implemented a system of aggressive ED triaging to separate patients with respiratory symptoms from other ED patients. Patient census in the ED decreased (Figure 3), enabling increased separation of patient gurneys.

The HCWs in the ED and intensive care unit were screened for MERS-CoV with RT-PCR (number screened unknown). Fourteen were positive and isolated at home; 13 of these remained asymptomatic and returned to work when they became RT-PCR negative (these 13 were not included as cases because they were asymptomatic). One positive HCW became ill while in home isolation, was hospitalized, and is included among the 78 cases.

Before the outbreak, MERS inpatients were isolated or cohorted in standard patient rooms, but infection control adherence was reportedly incomplete. Starting in week 8 (April 20–26), inpatients with known or suspected MERS were treated in 1 of 17 single-patient negative-pressure isolation rooms in a new hospital building; visitors were not allowed in patient rooms. Also during week 8, KFGH was designated a MERS treatment facility and began accepting transfers of MERS patients from other Jeddah hospitals. Starting May 9, KFGH leadership made additional changes, including implementation of a new MOH infection prevention and control guideline and management algorithm (*16*,*17*) that included a hospital-based clinical guideline with detailed procedures for treating MERS patients. Transport pathways, elevators, a radiograph room, and a resuscitation room were designated for MERS patient use.

Before the outbreak, dialysis was performed 24 hours/day during 6 days/week. During week 8 (April 20–26), after a visit from Saudi MOH officials, changes were made: dialysis was reduced from 4 to 3 shifts per day, space between patients was increased to a minimum of 1.2 m, and use of personal protective equipment (gowns, gloves, and surgical masks) among HCWs was required. None of the dialysis unit HCWs developed MERS during the outbreak period.

Discussion

Our results suggest that 78 persons (53 patients, 16 HCWs, 9 visitors) became symptomatically infected with MERS at KFGH during spring 2014. Among the patients, infection was acquired in multiple settings, including the ED (22 cases), inpatient areas (17 cases), outpatient dialysis unit (11 cases), and other outpatient areas (3 cases). We were able to directly track transmission (i.e., an infectious and a susceptible patient in the same room) only to 5 cases: 2 in inpatient areas and 3 in the

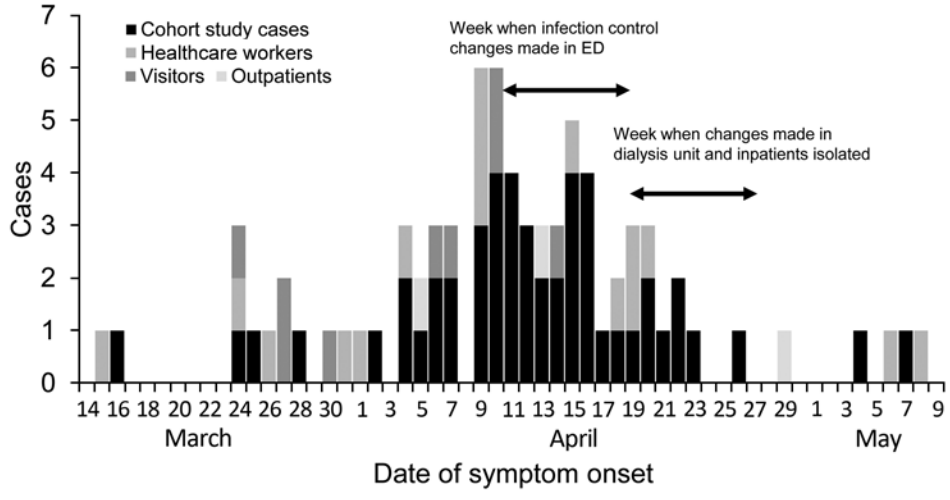

Figure 2. Middle East respiratory syndrome cases acquired at King Fahd General Hospital, by date of symptom onset, Jeddah, Saudi Arabia, March 2–May 10, 2014.The 78 patients comprised 50 cohort study cases, 16 healthcare workers, 9 visitors, and 3 outpatients. ED, emergency department.

Table 5. Retrospective cohort study of risk factors for Middle East respiratory syndrome coronavirus infection acquired at King Fahd General Hospital, Jeddah, Saudi Arabia, March 2–May 10, 2014

Population or variable	No. cases	No. patient-days	Rate*	Rate ratio	p value
Emergency department, inpatient areas, dialysis unit†	50	81,987	6.1		
Setting					
Emergency department	22	34,897	6.3	1.2	0.64
Inpatient areas	17	26,186	6.5	1.2	0.60
Dialysis unit	11	20,904	5.3	Referent	
Outbreak week: start date					
1: March 2	1	8,609	1.2	Referent	
2: March 9	0	11,136	0.0	Referent	
3: March 16	2	11,437	1.7	Referent	
4: March 23	5	11,793	4.2	4.4	0.047
5: March 30	11	10,666	10.3	10.7	<0.001
6: April 6	20	6,805	29.4	30.5	<0.001
7: April 13	8	5,654	14.2	14.7	<0.001
8: April 20	1	5,333	1.9	1.9	0.57
9: April 27	2	5,509	3.6	3.8	0.19
10: May 4	0	5,047	0.0	0.0	0.63
Emergency department, hospital inpatient areas‡	39	61,083	6.4		
Nationality					
Saudi	18	40,384	4.5	Referent	
Non-Saudi	21	20,698	10.1	2.3	0.01
Sex					
F	10	21,897	4.6	Referent	
M	29	39,151	7.4	1.6	0.18
Age group, y					
0–19	8	33,005	2.4	Referent	
20–39	10	16,888	5.9	2.4	0.064
40–59	18	9,400	19.1	7.9	<0.001
≥60	3	1,725	17.4	7.2	0.016

*Per 10,000 patient-days.
†For these analyses, cases were attributed to the setting or week that was most probable on the basis of incubation period (Tables 1, 2); p values do not reflect uncertainty caused by these attributions.
‡Denominator data for dialysis patients was not available; dialysis patients are not included in these analyses.

dialysis unit. However, most of these persons had several days (median 6.5) of treatment in KFGH before MERS onset (Figure 1), strongly suggesting that they acquired MERS at KFGH. In addition, we were able to determine setting-specific infection rates and found that they were similar in the ED, inpatient areas, and dialysis unit, indicating that risk for acquisition was widespread. Treatment in >1 setting of many of these patients complicated determination of these setting-specific rates. However, the 2 methods we used to overcome this problem, most likely site of acquisition and maximum-likelihood modeling, provided good agreement.

In previous MERS outbreaks, dialysis units have been implicated as a focus of disease transmission. At KFGH the patient-day risk for transmission in the dialysis unit was no greater than in the KFGH ED and inpatient areas; only 11 of the 22 cohort patients receiving dialysis were believed to have acquired MERS in the dialysis unit.

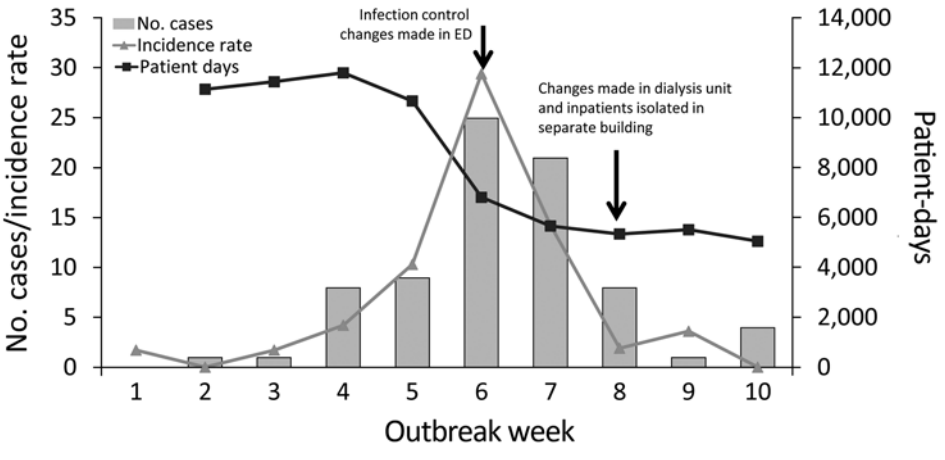

Figure 3. Number of Middle East respiratory syndrome cases, incidence rate, and number of patient-days of treatment at King Fahd General Hospital, Jeddah, Saudi Arabia, March 2–June 14, 2014. Incidence rate is cases per 100,000 patient-days. ED, emergency department.

Although MERS incidence rates were similar in the ED, inpatient areas, and dialysis unit, the ED was the most frequently implicated in disease acquisition among patients. Contributing factors probably included lack of triage, lack of isolation for patients with respiratory symptoms, extended stays for certain patients, and close proximity among patients. The KFGH outbreak peaked during week 6 and decreased sharply after week 7 (Figures 2, 3) after infection control improvements in the ED during week 6. During week 8, infection control was improved in the dialysis unit, and a MERS unit was established in a separate building. These improvements, in combination with decreased patient volume by week 6, probably led to decreased transmission.

The median onset date for KFGH-acquired MERS-CoV infection (April 11) was ≈2 weeks before that of cases that were not KFGH-acquired (April 22) and 1 week before all symptomatic cases in Jeddah (US Centers for Disease Control and Prevention, unpub. data). Thus, as indicated in a recent laboratory-based study (*15*), KFGH appears to have been an early focus of transmission in the Jeddah outbreak. Our cohort study reported higher incidence among older patients at KFGH, suggesting that clinically recognized disease did not develop in certain younger patients who were exposed to MERS-CoV infection at KFGH. Although we did not study HCW-to-patient transmission, asymptomatic HCWs were documented and might have contributed to disease transmission. The potential for transmission by asymptomatic persons needs further investigation.

Limitations to this study include incomplete clinical data for patients and for HCWs. In certain instances, we had to use clinical judgement to estimate disease onset dates. No records were kept on ED rooms in which patients were treated. Although we attempted to identify all non-KFGH healthcare exposures, certain exposures might not have been identified. We assumed that patients treated at KFGH at any time during the 2–14 days before illness onset had potentially KFGH-acquired disease. Records were not uniformly available when patients from other dialysis units or from the ED were dialyzed temporarily at KFGH; our results might not reflect disease transmitted from, or acquired by, these patients. The number of dialysis patient-days of treatment could only be estimated. Finally, data on genetic typing of MERS-CoV viruses from KFGH cases that could have helped define chains of transmission was not available to us.

In conclusion, the MERS outbreak at KFGH affected patients and HCWs. Illness most likely was transmitted in multiple settings, making it difficult to track disease from patient-to-patient. The contribution to MERS-CoV transmission at KFGH by asymptomatic persons is uncertain; transmission by asymptomatic persons is an area that needs further study. Heightened awareness of MERS, aggressive triaging of patients, prompt isolation, and strict infection control measures were associated with a rapid decrease in transmission. Continued vigilance and consistent adherence to infection control precautions is necessary to prevent future healthcare-acquired MERS outbreaks.

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At the time of the investigation, Dr. Hastings was an Epidemic Intelligence Service officer at the Centers for Disease Control and Prevention, assigned as a field officer; she currently works in the Division for Unintentional Injury Prevention, National Center for Injury Prevention and Control, CDC. Her research interests include injury prevention and environmentally caused illnesses.

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Expansion of Shiga Toxin–Producing *Escherichia coli* by Use of Bovine Antibiotic Growth Promoters

Jong-Chul Kim, Linda Chui, Yang Wang, Jianzhong Shen, Byeonghwa Jeon

Antibiotics are routinely used in food-producing animals to promote growth and prevent infectious diseases. We investigated the effects of bovine antibiotic growth promoters (bAGPs) on the propagation and spread of Shiga toxin (Stx)–encoding phages in *Escherichia coli*. Co-culture of *E. coli* O157:H7 and other *E. coli* isolated from cattle in the presence of sublethal concentrations of bAGPs significantly increased the emergence of non-O157, Stx-producing *E. coli* by triggering the SOS response system in *E. coli* O157:H7. The most substantial mediation of Stx phage transmission was induced by oxytetracyline and chlortetracycline, which are commonly used in agriculture. bAGPs may therefore contribute to the expansion of pathogenic Stx-producing *E. coli*.

Antimicrobial agents are commonly used subtherapeutically as feed supplements to promote growth and to prevent infection in livestock. Despite growing public health concerns about resistance associated with agricultural use of antibiotics, their use in livestock production is anticipated to increase (*1*). Each year in the United States, 1,675 tons of nontherapeutic antibiotics are used in beef cattle, particularly in feedlots, which are intensive cattle-raising systems (*2*). According to a report from the US Department of Agriculture, ionophores, tylosin, chlortetracycline, and oxytetracycline are frequently given to feedlot cattle (*3*). Ionophores, such as monensin, are included in feed mainly to increase weight gain and to prevent bovine coccidiosis (*3*,*4*). Tylosin is used to prevent diseases (e.g., hepatic abscessation) and to promote growth in cattle (*3*,*5*), whereas chlortetracycline and oxytetracycline are used as feed supplements mainly to prevent bovine pneumonia and bacterial enteritis (*4*). Although antibiotics are usually added to feed, water, or both at subtherapeutic levels, at some feedlots, chlortetracycline and oxytetracycline are used at therapeutic levels to prevent infection, particularly when calves are first introduced into feedlots (*6*).

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Shiga toxin–producing *Escherichia coli* (STEC), such as *E. coli* O157:H7, is the leading cause of hemorrhagic colitis and hemolytic uremic syndrome (*7*). Shiga toxin (Stx) is reportedly produced by ≈250 different O serotypes of *E. coli*; non-O157 STEC infection is becoming increasingly prevalent, accounting for up to 20%–50% of STEC infections in the United States (*8*). In particular, 6 serogroups (O26, O45, O103, O111, O121, and O145) are responsible for 83% of all non-O157 infections in the United States (*9*). The *stx* genes are encoded by lambdoid bacteriophages (*10*). Because secretion systems for Stx are lacking, the release of Stx is mediated through bacterial cell lysis by Stx phages in response to the induction of the SOS response (a cellular response to DNA damage) (*11*). Antimicrobial agents, particularly those that interfere with DNA synthesis (e.g., quinolones and trimethoprim), enhance the propagation of Stx phages and consequently increase Stx production (*12*). For this reason, antimicrobial drug treatment is not recommended for patients with enterohemorrhagic *E. coli* infection (*13*). In contrast, antibiotics are widely used as feed supplements in cattle, which are the primary natural reservoir for O157 and non-O157 STEC strains (*14*,*15*). *E. coli* is highly prevalent in cattle feces at levels ranging from 10^7 to 10^9 CFU/g (*16*), and *E. coli* O157 primarily colonizes the terminal rectum in cattle and is found in cattle feces at 10^3–10^5 CFU/g (*17*). Unlike humans, cattle are not susceptible to STEC infection because they lack Stx receptors (*18*); thus, antibiotics do not generate clinical problems in cattle. However, bovine antibiotic growth promoters (bAGPs) may induce the propagation of Stx phages and consequently facilitate the horizontal transfer of *stx* genes in *E. coli*. In this study, we investigated whether bAGPs can affect the propagation of Stx phages and contribute to the diversification of Stx-producing *E. coli*.

Materials and Methods

E. coli Strains, Plasmids, and Culture Conditions

We routinely maintained *E. coli* O157:H7 EDL933 and all *E. coli* isolates from cattle in Luria Bertani (LB) medium. The plasmid u66recA, a transcriptional fusion of *recA*::*egfp*,

is described elsewhere (*19*). Detoxified EDL933 strains (Δ*stx*2::*Km* and P_{stx2}::*gfp* in which *stx2* is replaced with a kanamycin resistance cassette and *gfp*, respectively) were constructed according to a method described by Datsenko and Wanner (*20*). The *stx*2 promoter region was PCR amplified from *E. coli* EDL933 with Pro_Stx2-F and Pro_Stx2-R primers (Table 1). The resulting 377-bp PCR product was purified, digested with *Xba*I, and ligated to an *Xba*I site located immediately upstream of the promoterless *gfp* gene in pFPV25.1 (*23*). P_{stx2}::*gfp* was prepared by PCR with GFP_BGL_F and GFP_BGL_R (Table 1). The PCR product was cloned to a *Bgl*II site upstream of the flippase recognition target (FRT) in pKD13 (*20*). The FRT-flanked P_{stx2}::*gfp* was amplified with PCR from pKD13 by use of pKD13-F and pKD13-R primers, and the amplicon was introduced to EDL933 harboring pKD46 by electroporation. The transcriptional P_{stx2}::*gfp* fusion was constructed by replacing the *stx*2 gene with *gfp* in EDL933. The pKD46 plasmid was cured from the mutant by culturing at 37°C. A Δ*stx*2::*Km* mutant of *E. coli* O157:H7 EDL933 was constructed by replacing *stx*2 with a FRT-flanked kanamycin resistance cassette that had been PCR amplified from pKD13. The FRT-*Km*-FRT amplicon was introduced into EDL933 harboring pKD46 by electroporation (*20*). The allelic exchange was confirmed by PCR with the primer sets of Stx2-F and Stx2-R, Kt and K1. We added tetracycline (50 μg/mL), ampicillin (100 μg/mL), and kanamycin (50 μg/mL) to culture media when necessary.

Antibiotics

Monensin, tylosin, chlortetracycline, oxytetracycline, neomycin, and sulfamethazine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ciprofloxacin was purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA).

Measurement of P_{stx2}::*gfp* Expression

Cultures carrying the P_{stx2}::*gfp* promoter fusion were collected in the exponential phase by centrifugation (5 min at 6000 × *g*), and bacterial cells were resuspended in fresh LB medium to ≈4.0 × 10^7 CFU/mL. After growing in LB broth supplemented with various concentrations of bAGPs and ciprofloxacin for 3 h, 200 μL samples were transferred to each well in a 96-well microplate and green fluorescent protein wavelength was measured with a fluorometer (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). Fluorescence was monitored at excitation and emission wavelengths of 520 nm and 480 nm, respectively. Fluorescence intensities are reported in the instrument's relative fluorescence units. The expression level of *rec*A was measured in the same way with *u66rec*A. The experiments were performed with triplicate samples and repeated at least 3 times.

Stx Phage Induction

Stx phages were induced by monensin, tylosin, chlortetracycline, oxytetracycline, and ciprofloxacin. Because ciprofloxacin is a well-known Stx phage inducer (*12*), we used ciprofloxacin as a control. Lysogenic strains were grown in LB broth overnight at 37°C with shaking and diluted to an optical density at 600 nm of 0.1 in NZCYM broth (Amresco, Solon, OH, USA). The cultures were incubated at 37°C with shaking (200 rpm) for 18 h in the presence (0.01, 0.1, and 1 μg/mL) and absence (control) of the 5 antibiotics. After centrifugation at 5,000 × *g*, the supernatant was sterilized with a 0.22-μm filter and used immediately. We then added 10-fold serial dilutions of phage lysates to 1 mL of *E. coli* C600 at the stationary phase. Then, 3 mL of top agar supplemented with 5 mmol/L calcium chloride was added to this culture and the mixture was poured on an LB agar plate. The plates were incubated at 37°C overnight, and PFU were counted the next day.

Stx Phage Transfer Assay

We investigated the transfer of Stx phages in the presence of bAGPs at different concentrations. Briefly, 5 mL of *E.*

Table 1. Primers used in study of expansion of Shiga toxin–producing *Escherichia coli* by bovine antibiotic growth promoters

Primer	Sequence, 5′ → 3′	Reference
Pro_Stx2-F	TAAGCATCTAGATTGCAGGATTAGTTACGT	This study
Pro_Stx2-R	TGCTTATCTAGAACAGGTGTTCCTTTTGGC	
GFP_BGL_F	TTCGAGCTCAGATCTCGGGGATCC	This study
GFP_BGL_R	TGCTTAAGATCTCGCATTAAAGCTTGCATG	
pkD13-F	CCAGGCTCGCTTTTGCGGGCCTTTTTTAT	This study
pkD13-R	GTGACACAGATTACACTTGTTACCCACAT	
Kt	CGGCCACAGTCGATGAATCC	(*20*)
K2	CGGTGCCCTGAATGAACTGC	
Stx2-F	GTCTGGTGCTGATTACTTCAGCCAA	This study
Stx2-R	ATTACACTTGTTACCCACATACCAC	
eaeA-F	GACCCGGCACAAGCATAAGC	(*21*)
eaeA-R	CCACCTGCAGCAACAAGAGG	
Ec1_uspA	CCGATACGCTGCCAATCAGT	(*22*)
Ec2_uspA	ACGCAGACCGTAAGGGCCAGAT	
O157F	CGGACATCCATGTGATATGG	(*21*)
O157R	TTGCCTATGTACAGCTAATCC	

Table 2. MICs and MBCs of bAGPs for *Escherichia coli* O157:H7 EDL 933

bAGP*	MIC, μg/mL	MBC, μg/mL
Monensin	>1,024	>1,024
Tylosin	>1,024	>1,024
Chlortetracycline	4	8
Oxytetracycline	8	16

*bAGP, bovine antibiotic growth promoter; MBC, minimum bactericidal concentration.

coli EDL933 Δ*stx*2::*Km* (donor) and 6 *stx*2-negative bovine *E. coli* strains (recipients) with ampicillin or tetracycline resistance were grown in LB broth at 37°C overnight. Overnight cultures were diluted 100-fold in fresh NZCYM broth and cultured until the early exponential phase for 3 h. The donor strain (≈10^4 CFU/mL) was mixed with recipient strains (≈10^7 CFU/mL) in the presence of different concentrations of bAGPs. The mixed cultures were incubated at 37°C overnight without shaking. After incubation, 100 μL of culture was spread onto sorbitol-MacConkey agar plates supplemented with kanamycin and ampicillin or tetracycline and incubated at 37°C overnight. We calculated the transduction frequencies by dividing the number of transductants by the number of recipients.

Characterization of Transductants

Pink colonies growing on sorbitol-MacConkey agar supplemented with either kanamycin and tetracycline or kanamycin and ampicillin were regarded as presumptive transductants (i.e., recipients of the *stx*2-encoding phage 933W). The presumptive transductants of the *stx*2-encoding phage 933W were verified by performing multiplex PCR. PCRs were performed with specific primer pairs: Stx2-F and Stx2-R for the *stx*2 gene in 933W, eaeA-F and eaeA-R for *eae*A encoding intimin (*21*), Ec1-uspA and Ec2-uspA for the *usp*A gene encoding the universal stress protein in *E. coli* (*22*), and O157F and O157R for a region in *rfb*E (O-antigen-encoding) for the O157 serotype (*21*). Serologic tests were performed with O157 and O26 antiserum (Korea National Institute of Health, Osong, South Korea).

Results

Enhanced Propagation of Stx Phages in *E. coli* O157:H7 by bAGPs

To investigate the effect of subtherapeutic concentrations of bAGPs on the propagation of Stx phages, we first measured the level of Stx phage propagation after exposure of *E. coli* O157:H7 EDL933 to sublethal concentrations (1, 0.1, and 0.01 μg/mL) of common bAGPs, including monensin, tylosin, chlortetracycline, and oxytetracycline. Because the bAGP concentrations used in the study were markedly less than the MICs (Table 2), bAGP treatment did not affect the growth of *E. coli* O157 (data not shown). The propagation of Stx phages was induced significantly by chlortetracycline and oxytetracycline at concentrations as low as 0.01 μg/mL (Figure 1, panel A). Because *E. coli* O157:H7 EDL933 harbors 2 Stx prophages, BP-933W (*stx*2) and CP-933V (*stx*1) (*24*), the level of *stx*2 expression was specifically measured with an *stx*2::*gfp* fusion construct to determine the propagation level of Stx2 phage. Consistently, bAGP treatment substantially increased the level of *stx*2 expression (Figure 1, panel B). Because the primary mechanism for antibiotic-mediated

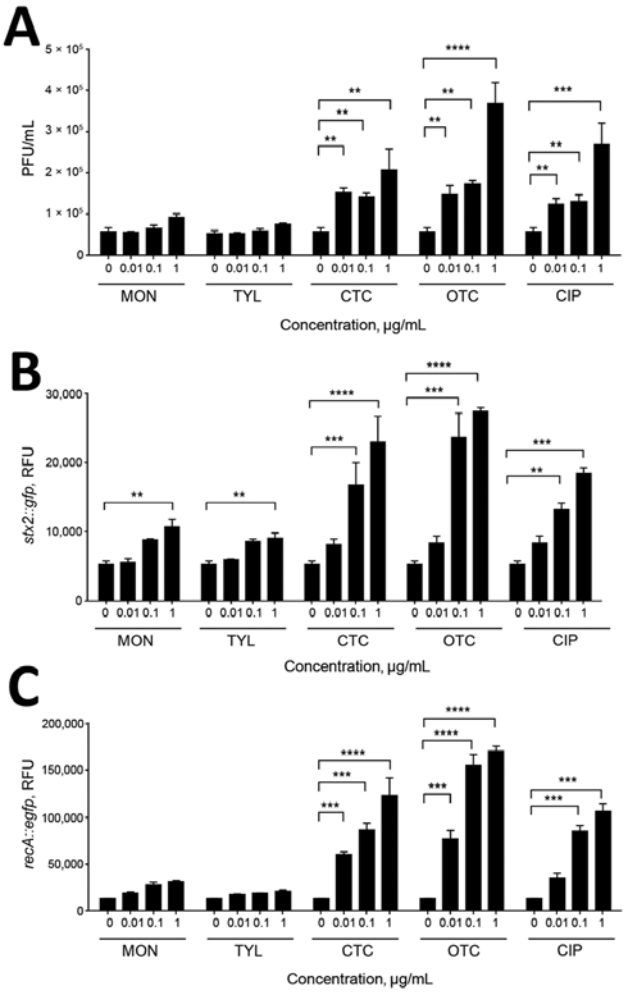

Figure 1. Induction of Shiga toxin (Stx) 2 phage propagation and SOS response by bovine antibiotic growth promoters (bAGPs). A) Stx2 phage induction in *Escherichia coli* O157:H7 EDL933 after 3 h exposure to subtherapeutic concentrations of bAGPs, including monensin (MON), tylosin (TYL), chlortetracycline (CTC), and oxytetracycline (OTC). Ciprofloxacin (CIP) was a control for phage induction. *E. coli* C600 was used as a phage-susceptible strain. B) Induction of *stx*2 expression by bAGPs. Fluorescence from an *stx*2::*gfp* transcriptional fusion indicates the level of Stx2 phage induction. C) Induction of *recA* transcription by bAGPs. The level of *recA* expression indicates the level of SOS response induction. The results show means and SDs of 3 independent experiments. Statistical significance was analyzed by using the Student *t*-test. **p<0.01, ***p<0.001, ****p<0.0001.

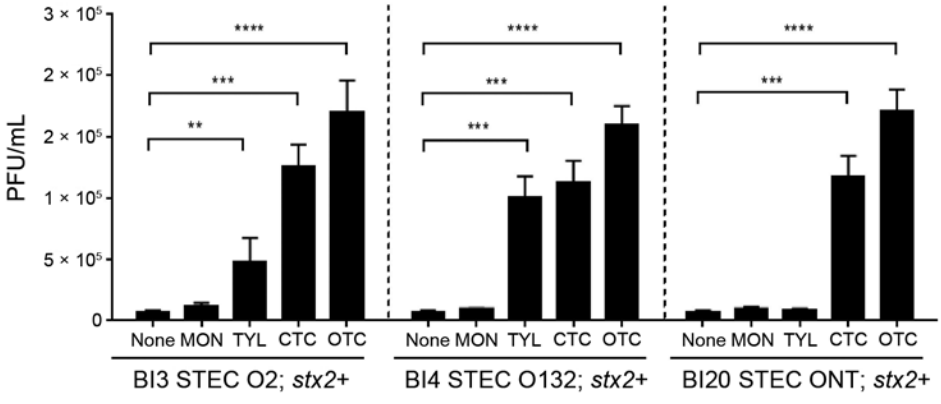

Figure 2. Induction of Shiga toxin (Stx) 2 by bovine antibiotic growth promoters (bAGPs) in Shiga toxin–producing *Escherichia coli* (STEC) strains from cattle. The levels of Stx2 phage induction were examined with 0.1 μg/mL monensin (MON), tylosin (TYL), chlortetracycline (CTC), and oxytetracycline (OTC). ONT, O antigen nontypable. The results show means and SDs of 3 independent experiments. Statistical significance was analyzed by using the Student *t*-test. **p<0.01, ***p<0.001, ****p<0.0001.

induction of phage propagation is the SOS response, we also determined the level of *rec*A expression after exposure to bAGPs. Consistent with the changes in the level of Stx phage propagation (Figure 1, panels A, B), chlortetracycline and oxytetracycline significantly induced *recA* expression (Figure 1, panel C). Of note, chlortetracycline and oxytetracycline induced Stx phage propagation and *stx*2 expression at levels similar to those of ciprofloxacin, a DNA-damaging antibiotic frequently used as a phage inducer (Figure 1).

Increased Propagation of Stx Phages in Bovine STEC Strains by bAGPs

When we further examined Stx phage induction by bAGPs with 3 *stx*2+/*stx*1– *E. coli* strains from cattle, we found that exposure to a sublethal concentration (0.1 μg/mL) of chlortetracycline and oxytetracycline significantly induced the propagation of Stx2 phage in the bovine STEC isolates, whereas 0.1 μg/mL of monensin did not induce phage propagation, and 0.1 μg/mL of tylosin exhibited strain-dependent variations in the phage induction (Figure 2).

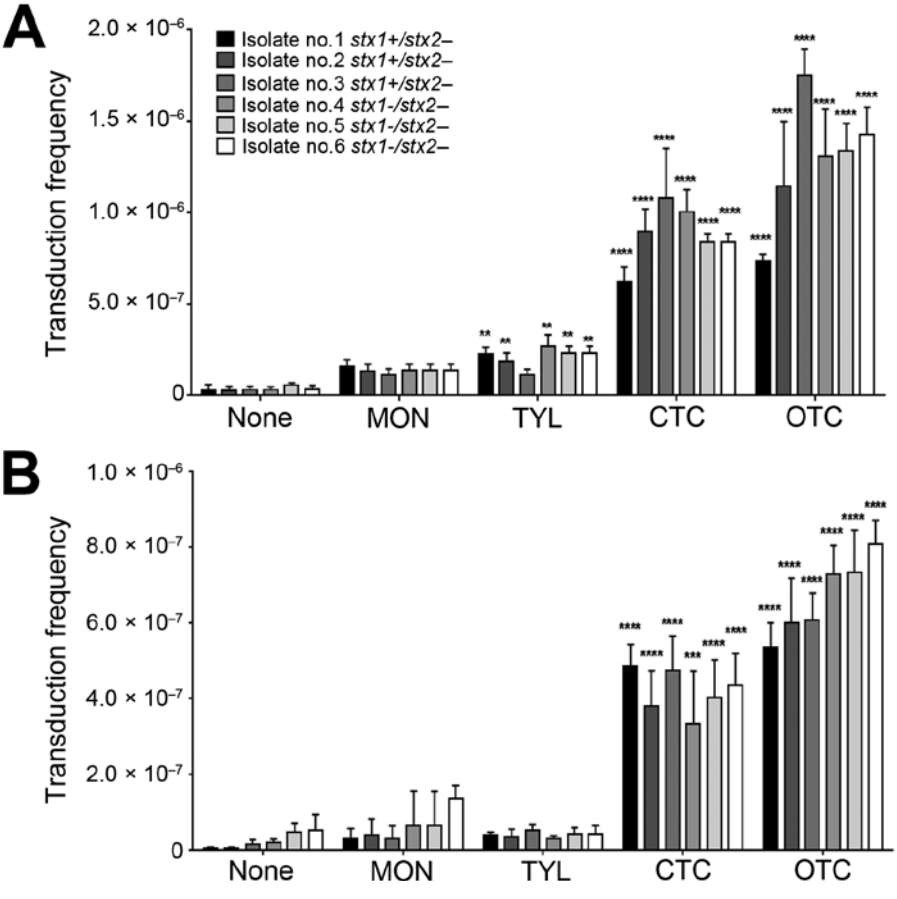

Figure 3. Emergence of Shiga toxin (Stx) 2–positive strains of *Escherichia coli* from cattle (isolates 1–6) by subtherapeutic bovine antibiotic growth promoters (bAGPs) at 0.1 μg/mL (A) and 0.01 μg/mL (B). Frequency of transfer of Stx phages to bovine *E. coli* isolates. The Stx phages originated from a detoxified derivative of *E. coli* O157:H7 EDL933, where *stx2* was replaced with a kanamycin resistance cassette. The donor and recipient *E. coli* strains were co-cultured with or without monensin (MON), tylosin (TYL), chlortetracycline (CTC), and oxytetracycline (OTC). The transduction frequencies were calculated by dividing the number of transductants by the number of recipients. The results show means and SDs of 3 independent experiments. Statistical significance was analyzed by using the Student *t*-test in comparison with antibiotic-free cultures. **p<0.01, ***p<0.001, ****p<0.0001.

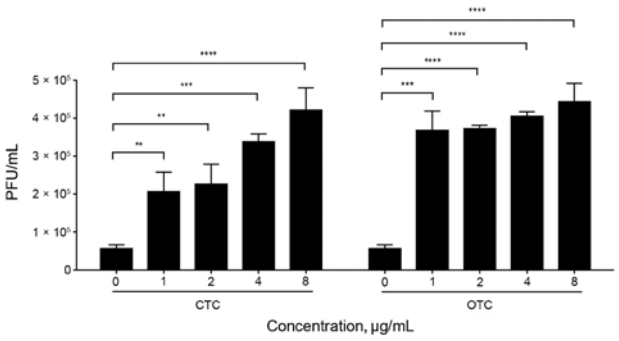

Figure 4. Induction of Stx2 phages by treatment with high concentrations of chlortetracycline (CTC) and oxytetracycline (OTC). The phage titer was determined in *Escherichia coli* O157:H7 EDL933 by treatment with 1 to 8 μg/mL of CTC and OTC. The results show means and SDs of 3 independent experiments. Statistical significance was analyzed by using the Student *t*-test in comparison with antibiotic-free cultures. **p<0.01, ***p<0.001, ****p<0.0001.

Transfer of Stx Phages in Bovine *E. coli* Isolates by Sublethal Concentrations of bAGPs

We determined the frequency of Stx2 phage transfer with 6 *stx*2-negative *E. coli* isolates from cattle, including 3 *stx*1+/*stx*2– *E. coli* strains and 3 *stx*1–/*stx*2– *E. coli* strains, by using a detoxified EDL933 derivative in which *stx*2 was replaced with a kanamycin resistance cassette. The donor *E. coli* (EDL933 Δ*stx*2::*Km*, a detoxified strain) and the recipient *stx2*-negative *E. coli* strains were co-cultivated in the presence of sublethal concentrations (0.01 μg/mL and 0.1 μg/mL) of bAGPs. The recipient bovine *stx*2-negative *E. coli* isolates are all sensitive to kanamycin and resistant to β-lactams or tetracycline, and the detoxified EDL933 derivative is resistant to kanamycin and sensitive to β-lactams and tetracycline. Therefore, the Stx phage transfer made the recipient strains resistant to both kanamycin and β-lactams or tetracycline. Subtherapeutic treatment of bAGPs, particularly chlortetracycline and oxytetracycline, substantially enhanced the transfer of Stx2 phage in *E. coli* (Figure 3, panel A). The transduction rate was slightly increased by 0.1 μg/mL tylosin but not notably affected by 0.1 μg/mL monensin (Figure 3, panel A). When the concentration of bAGPs was reduced to 0.01 μg/mL, tylosin did not mediate the Stx phage transfer. However, chlortetracycline and oxytetracycline significantly mediated the transfer of Stx phage in *E. coli* even at 0.01 μg/mL (Figure 3, panel B).

Induction of Stx Phage Propagation by Therapeutic Concentrations of Chlortetracycline and Oxytetracycline

Whereas tylosin and monensin are used at low concentrations in cattle feed, chlortetracycline and oxytetracycline are sometimes used at therapeutic levels to prevent infection (*6*). Thus, we investigated the effects of high concentrations of chlortetracycline and oxytetracycline on the propagation of Stx phages. High concentrations of chlortetracycline significantly increased the propagation of Stx phages in a concentration-dependent manner, whereas the level of Stx phage induction by oxytetracycline is already significantly high at 1 μg/mL in comparison with higher concentrations of oxytetracycline (2–8 μg/mL) and even the highest concentration of chlortetracycline used in the study (8 μg/mL) (Figure 4). These findings demonstrate that therapeutic application of chlortetracycline may enhance the dissemination of Stx phages more significantly than subtherapeutic doses and that oxytetracycline is a highly potent inducer of Stx phage propagation even at low concentrations.

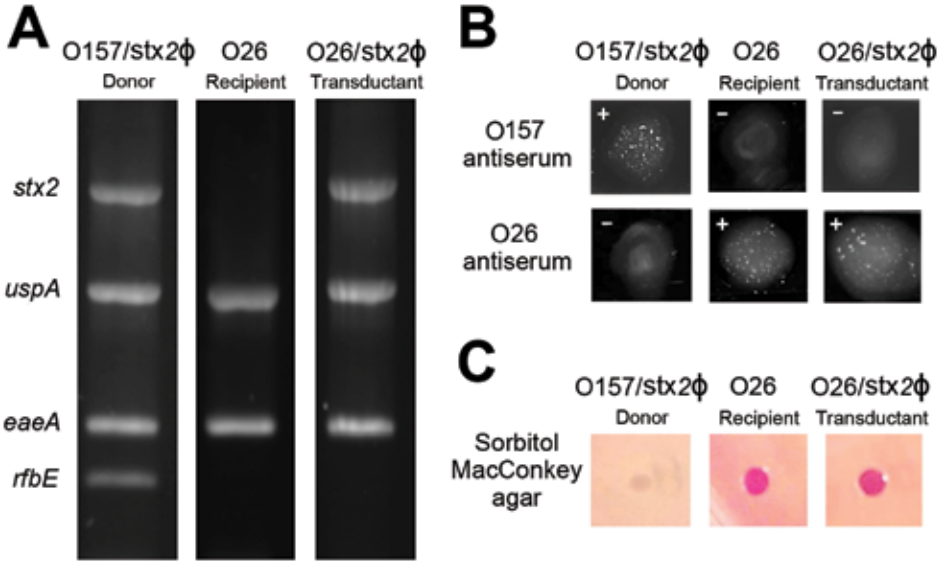

Figure 5. Transfer of Shiga toxin (Stx) phages by bovine antibiotic growth promoters (bAGPs) in *Escherichia coli* isolates from cattle**.** For the confirmation of Stx phage (Stx2Φ) transfer, *E. coli* O26 (*stx2*-negative, bovine isolate no. 1 in Figure 3) was used. A) PCR confirmation of the presence or absence of *stx2*, *usp*A, *eae*A, and *rfb*E_{O157} in EDL933 (Stx2Φ donor), O26 before transduction (Stx2Φ recipient), and O26 after transduction (transductant). B) Serotyping of *E. coli* O157 and O26. +, positive reaction; –, negative reaction. C) Sorbitol fermentation of donor, recipient, and transduced strains on sorbitol MacConkey agar plates. *E. coli* O157 does not ferment sorbitol, whereas non-O157 *E. coli* ferments sorbitol and produces pink colonies.

Confirmation of Stx Phage Transfer by bAGPs

To confirm Stx phage transfer by bAGPs, we randomly chose transductant colonies from the co-culture experiment described earlier (Figure 3) for further verification with PCR to detect genes specific for the Stx2 phage (*stx*2), *E. coli* (*usp*A), *E. coli* virulence (*eae*A), and O157 serotype ($rfbE_{O157}$) (Figure 5, panel A). Figure 5 shows representative data to exhibit the dissemination of the *stx2* gene to *stx*2-negative *E. coli* O26 (bovine isolate no. 1 in Figure 3) by exposure to bAGPs. We selected *E. coli* O26 because this serotype is the most frequently isolated non-O157 STEC (*9*). Treatment with bAGP changed bovine *E. coli* O26 from *stx*2-negative to *stx*2-positive. We also performed a latex agglutination test to confirm the serotype after transduction (Figure 5, panel B). The capability of sorbitol fermentation in non-O157 strains was confirmed by growing on sorbitol MacConkey agar plates (Figure 5, panel C). The results clearly showed that non-O157 *E. coli* horizontally acquired *stx*2 by phage transduction after exposure to bAGPs.

Discussion

In livestock production, antibiotics are routinely added to feed for growth promotion and disease prevention. Although these AGPs are used at subtherapeutic concentrations, a substantial number of studies have shown that AGPs may negatively affect public health by providing selective pressure to increase antibiotic-resistant pathogens (*25*,*26*). Our study showed that, in addition to growing public health concerns about antibiotic resistance, some AGPs may facilitate the transmission of virulence factors in *E. coli* even at extremely low concentrations. Whereas the effect of monensin and tylosin on the propagation of Stx phages seemed to be marginal, chlortetracycline and oxytetracycline significantly induced the propagation of Stx phages (Figures 1, 2, 4) and mediated the transfer of Stx phages in *E. coli* (Figure 3).

Tetracyclines are widely used in agriculture, accounting for 44% and 37% of marketed agricultural antibiotics in the United States (*27*) and the European Union (*28*), respectively. Compared with monensin and tylosin, oxytetracycline and chlortetracycline most significantly affected the transmission of Stx phages, even at concentrations as low as 0.01 μg/mL (Figure 3, panel B). This concentration is substantially lower than concentrations in the large intestines of cattle, which are 0.3 μg/mL after chlortetracycline feeding for growth promotion (70 mg/head/day throughout feedlot period) and 1.7 μg/mL after feeding for disease prevention (350 mg/head/day for 28 days) (*29*). A previous observational study reported that the percentage of detecting *stx*-positive commensal *E. coli* was increased in cattle from 48% to 80% by oxytetracycline injection and chlortetracycline addition to feed (*30*). Although they did not conclusively say that the increase in *stx*-positive animals is from oxytetracycline and chlortetracycline treatment in cattle, they suggested that antibiotic treatment may be the reason for the increased prevalence (*30*). Sometimes, chlortetracycline and oxytetracycline are mixed with other antibiotics, such as neomycin and sulfamethazine, to maintain weight gains and feed efficiency for cattle under stress conditions (*4*). We observed that tetracycline combinations with these antibiotics induced Stx phage propagation just as comparably as a single treatment of chlortetracycline or

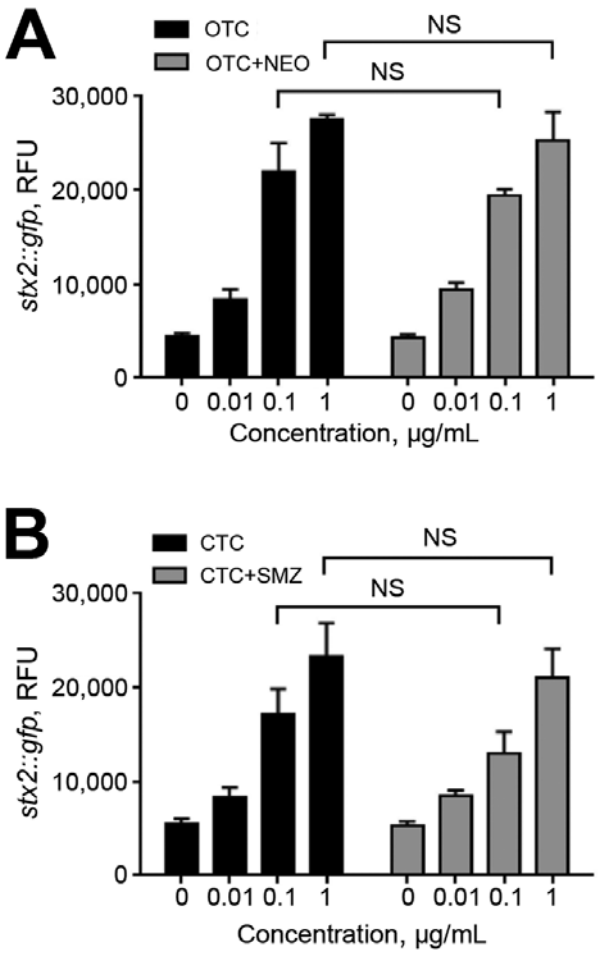

Figure 6. Induction of *stx2* expression by treatment of oxytetracycline (OTC) and chlortetracycline (CTC) in combination with other antibiotics. *Escherichia coli* O157 harboring P_{stx2}::*gfp* was exposed to the following antibiotic combinations that are used in cattle for weight gain and feed efficiency: A) OTC/neomycin (NEO), and B) CTC/sulfamethazine (SMZ). The antibiotic combinations were prepared by mixing the indicated concentrations of each antibiotic. The results show means and SDs of a single representative experiment with triplicate samples. The experiment was repeated 3 times, and similar results were observed in all experiments. Statistical analysis was performed by using a Student *t*-test and GraphPad Prism 6 (http://www.graphpad.com/). NS, not significant; RFU, relative fluorescence units.

oxytetracycline alone (Figure 6), suggesting that oxytetracycline and chlortetracycline are the major bAGPs that induce propagation of Stx phages. Another concern about bAGPs would be associated with poor absorption of orally administered antibiotics in animal guts (*31*). Approximately 75% of dietary chlortetracycline is excreted in cattle manure without being digested (*32*), and chlortetracycline is the antimicrobial compound that is most frequently detected in cattle manure at levels as high as 20 mg/kg (*33*). Given the high residue concentrations in mature, unmetabolized tetracycline residues may also affect the dissemination of Stx phages in cattle manure.

Although use of chlortetracycline and oxytetracycline in cattle is not consistent (*4*), the levels of SOS response induction and Stx phage propagation by these 2 antibiotics were comparable (Figure 1). In addition to subtherapeutic use in feed, therapeutic concentrations of oxytetracycline and chlortetracycline are sometimes added to feed as metaphylaxis in feedlot cattle (*6*). We observed that chlortetracycline induced propagation of Stx phages more significantly at high concentrations than at low subtherapeutic concentrations (Figure 4). Surprisingly, propagation of Stx phages by 1 μg/mL oxytetracycline was comparable to that of 8 μg/mL chlortetracycline (Figure 4), suggesting that oxytetracycline is highly effective in phage induction. Possibly, therapeutic administration of chlortetracycline, oxytetracycline, and other antibiotics, particularly those inducing the SOS response and Stx phage propagation (e.g., fluoroquinolones) (*12*), would significantly affect the spread of Stx phages; however, its effect would be limited because therapeutic antibiotics are usually used to treat disease in individual animals.

Previous studies have reported that antibiotic treatment significantly increases the propagation of Stx phages (*24, 34–36*). However, little attention has been paid to the effects of nonprescription bAGPs on the transmission of Stx phages in *E. coli*, although phages are a well-known vehicle for horizontal gene transfer and cattle are the primary reservoirs for *E. coli* O157:H7. Presumably, the underestimation of bAGPs might result from low concentrations of antibiotics in cattle feed. Nevertheless, in this study, we demonstrated that some bAGPs, particularly chlortetracycline and oxytetracycline, are implicated in the diversification of *stx*-positive O serotypes in *E. coli* by facilitating the horizontal transfer of Stx phages even at substantially low concentrations. Thus, use of these agents could lead to emergence of pathogenic *E. coli.*

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Acute Human Inkoo and Chatanga Virus Infections, Finland

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Inkoo virus (INKV) and Chatanga virus (CHATV), which are circulating in Finland, are mosquitoborne California serogroup orthobunyaviruses that have a high seroprevalence among humans. Worldwide, INKV infection has been poorly described, and CHATV infection has been unknown. Using serum samples collected in Finland from 7,961 patients suspected of having viral neurologic disease or Puumala virus infection during the summers of 2001–2013, we analyzed the samples to detect California serogroup infections. IgM seropositivity revealed 17 acute infections, and cross-neutralization tests confirmed presence of INKV or CHATV infections. All children (≤16 years of age) with INKV infection were hospitalized; adults were outpatients with mild disease, except for 1 who was hospitalized with CHATV infection. Symptoms included fever, influenza-like illness, nausea or vomiting, disorientation, nuchal rigidity, headache, drowsiness, and seizures. Although many INKV and CHATV infections appear to be subclinical, these viruses can cause more severe disease, especially in children.

Inkoo virus (INKV) and Chatanga virus (CHATV) are 2 members of the California serogroup of orthobunyaviruses that are currently found in Finland. They are trisegmented, enveloped negative-strand RNA viruses belonging to genus *Orthobunyavirus* (family *Bunyaviridae*), which includes several recognized mosquitoborne human pathogens.

INKV was first isolated from *Ochlerotatus communis* and *O. punctor* mosquitoes in 1964 in Finland (*1*) and has since been found in Sweden, Norway, and Russia (*2–4*). The high seroprevalence in these countries suggests that INKV infections are common in these locations (*5–8*). Although the virus has been known to occur in Finland for decades, only 1 domestic report describes a possible association of INKV to clinical disease (*9*). Reports from Russia show INKV IgM or neutralizing antibodies in patients with neurologic symptoms or fever, but only 2 cases were identified as INKV infection; most often, the California serogroup virus infections were caused by Tahyna virus (TAHV), or the infecting virus could not be defined (*10–12*).

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CHATV was isolated from mosquitoes collected in Finland in 2007 (*13*) but is known to have circulated earlier in Russia, where the first characterized isolate was from a mosquito collected in 1987 (*14*). CHATV strains have ≈84% aa identity with INKV within the nucleocapsid protein but are more similar to the Snowshoe hare virus (93% nucleocapsid protein identity) that occurs in the United States. We found no previous reports of CHATV infections naturally occurring in humans or animals.

Patients with California serogroup virus infections usually remain asymptomatic or have symptoms of mild influenza-like illness, but some of these viruses may also cause encephalitis (*15–17*). The California serogroup viruses cross-react on many serologic tests, so neutralization assays are required to verify the specific virus. In the United States, 29–167 cases of California serogroup virus encephalitis are diagnosed annually, and most cases result from La Crosse virus (LACV) (*18*), which is one of the most important arboviral agents causing encephalitis in children in the United States but is rarely found in adults. This pattern contrasts with the arbovirus West Nile virus, which causes central nervous system (CNS) infections in adults more often than in children (*19*). LACV encephalitis can be mistaken for herpes simplex virus (HSV) or enterovirus meningoencephalitis and is often undiagnosed (*15,20*). Other California serogroup viruses that cause neuroinvasive disease in the United States and Canada are California encephalitis, Jamestown Canyon, and Snowshoe hare viruses (*17,21–24*), although infections caused by these viruses are reported more rarely than those caused by LACV and are not as extensively studied.

The incidence of California serogroup virus infections in Europe is largely unknown because of underdiagnosing and underreporting that result from lack of alertness among healthcare workers and lack of surveillance efforts. Available data indicate that TAHV has the most widespread distribution in Europe and is mostly asymptomatic or causes febrile illness, especially in children (*16,25,26*).

Because pathogens that cause encephalitis during the summer months in Finland are mostly unknown (*27*), we attempted to study the occurrence of acute California serogroup virus infections, particularly those caused by INKV, in febrile and encephalitic patients during the mosquito season in Finland and to characterize those infections. We

report our observance of INKV and CHATV infections in humans and describe the clinical characteristics of acute infections caused by these viruses.

Methods and Materials

Patient Samples

Our analysis comprised 3 sets of patient samples (7,961 total patients). First, we retrospectively screened serum samples that were collected from patients in healthcare facilities across Finland during the summer months of 2001–2013 (Table 1) and were sent as diagnostic samples to the Department of Virology and Immunology, Helsinki University Central Hospital Laboratory, Hospital District of Helsinki and Uusimaa (institutional review board permit 119/E0/05). For patients presumed to have CNS symptoms, samples were screened for antibodies against a panel of meningoencephalitis agents (HSV, varicella zoster virus, human herpesvirus 6, enterovirus, and *Mycoplasma pneumoniae* bacteria); for patients presumed to have febrile illness, samples were screened for Puumala virus. For most samples, laboratory screening was negative for the viral agents studied. In addition to these 2 sample groups, we analyzed samples specifically received for screening of INKV antibodies during the study period.

Of the total 8,793 samples we tested for California serogroup virus IgM, 4,214 serum samples and 832 cerebrospinal fluid (CSF) samples had been initially sent for screening of meningoencephalitis agents; 3,574 serum samples had been initially sent for Puumala virus testing; and 173 serum samples had been sent for INKV testing. Samples were stored at -20°C; aliquots of the serum samples were stored at –70°C for PCR testing. Laboratory data and patient histories were collected from patient records for cases with confirmed California serogroup virus IgM positivity. Data on 2 previously confirmed cases with INKV infection that occurred in 1976 and 1980 were included in the analysis; these cases had been confirmed with hemagglutination inhibition and neutralization tests, and full patient histories had been described previously (*9*).

Serologic Testing

Serum samples were screened with an indirect INKV-IgM immunofluorescence (IFA) test described previously (*5*). In brief, IFA slides contained Vero E6 cells (green monkey kidney cells, American Type Culture Collection, CRL-1586, Manassas, VA, USA); 30% of the cells were infected with INKV. Samples (serum diluted at 1:20 ratio; CSF undiluted) were incubated on slides overnight and then washed with phosphate-buffered saline; anti–human IgM fluorescein isothiocyanate conjugate was then added, and samples were incubated for 1 hour. After being washed with phosphate-buffered saline, slides were dried and examined with a fluorescence microscope. IgM-positive samples were retested with IgM IFA after removal of IgG by using Gullsorb treatment (Meridian Bioscience, Inc.,

Table 1. Serum samples screened for California serogroup virus IgM and IgM-positive samples, by patient group and date of collection, Finland, 2001–2013*

Patient group and date of sample collection†	Serum samples, no.	IgM-positive samples, no.	IgM prevalence, %
Suspected Puumala virus infection			
2001 May 25–Sep 4	1,294	2	0.15
2004 Jun 14–Sep 1	958	1	0.10
2012 Jun 5–Aug 21	498	2	0.40
2013 May 16–Sep 26	824	0	0
Total	3,574	5	0.14
Neurologic symptoms			
2003 Jun 2–Sep 29	711	2	0.28
2004 Jun 10–Sep 17	868	2	0.23
2005 Jun 23–Oct 1	969	2	0.21
2007 Jun 20–Aug 30	563	0	0
2012 Jun 8–Oct 15	1,103	3	0.27
Total	4,214	9	0.21
Suspected Inkoo virus infection			
2004	32	1	3.13
2005	30	0	0
2006	21	0	0
2007	31	0	0
2008	11	0	0
2009	15	0	0
2010	16	0	0
2011	14	0	0
2012	3	0	0
Total	173	1	0.58
All patient groups	7,961	15	0.19

*Samples from patients were initially screened for Puumala virus, for agents causing neurologic infections, or Inkoo virus. Indirect immunofluorescence was used to screen for California serogroup virus IgM.
†Sample collection for suspected Inkoo virus infection was for the entire year.

Cincinnati, OH, USA) for the serum samples. To ensure that diagnostic criteria of acute infection were met, as previously described, IgM-positive samples were confirmed with IgG IFA testing (*5*) and studied with INKV prototype strain KN3641 (*1*), CHATV Möhkö strain M07–1 (*13*), and TAHV prototype strain Bardos 92 (*28*) plaque-reduction neutralization test (PRNT) (*13*). PRNT was performed because of the occurrence of cross-reactions.

Reverse Transcription PCR

When possible (i.e., when sufficient sample remained after serologic testing), IgM-positive samples were tested with reverse transcription PCR (RT-PCR) to study the presence and kinetics of viremia and to obtain information on the viral sequences. RNA extraction was performed by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The extracted RNA was reverse transcribed to cDNA with ERT-Ro Roche Expand Reverse Transcriptase (Roche, Indianapolis, IN, USA). PCR methods, as described previously (*29,30*), were modified to work with the Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, Grand Island, NY, USA). The mixture included 1.25 μL of each primer (10 μmol/L), 12.5 μL of the Phusion Flash 2X buffer, and 8 μL of sterile distilled deionized water, producing a total volume of 25 μL, which included 2 μL of cDNA.

Results

Our hospital diagnostic laboratory received 7,961 serum and 832 CSF samples from 7,961 patients in Finland during 2001–2013 (Table 1). Samples were initially submitted to determine antibodies against either a panel of agents causing neurologic infections (4,214 serum and 832 CSF samples), Puumala virus (3,574 serum samples), or INKV (173 serum samples) (Table 1). Most (4,299 [54%]) serum samples were from healthcare settings in southern Finland. Children <10 years of age had the smallest number of samples; adults 50–59 years of age had the largest number of samples. A slight preponderance (52%) of sampled patients were male.

Including the 2 previously confirmed cases, a total of 17 serum samples and no CSF samples were found IgM positive for California serogroup viruses. Serum samples were IgM positive among 0.21% of patients with CNS symptoms, 0.14% of patients with suspected Puumala virus infection, and 0.58% of patients with suspected INKV infection (Table 1). Frequency of IgM positivity was similar for different years of sample collection. The age range of patients with positive results was 7–81 years; 8 patients were female and 9 were male. California serogroup viruses IgM was found most commonly in patients <19 or 50–59 years of age (Table 2). Most (88%) IgM-positive cases were detected during or after late August. Fourteen (82%) of the 17 patients showed the highest neutralizing antibody titers (up to 1,280) against INKV, whereas 3 patients showed the highest titers (up to 20,480) against CHATV. Neutralization tests for 2 of the 3 CHATV patients resulted in titers >4 times those for other studied California serogroup viruses, which met the diagnostic criterion for confirmed CHATV infection; the third CHATV patient with a lower titer likely had CHATV infection (Table 3). The earliest that IgM was detectable was day 3 of symptom onset (fever); 1 patient still had detectable IgM 3 weeks after symptom onset (Table 4). IgM titers varied generally between 1:30 and 1:160, and exceeded 1:320 in only 3 patients. IgG in IFA was detected in most IgM-positive patients. No patient had detectable California serogroup virus RNA.

Clinical histories were collected for all patients whose samples had a positive California serogroup virus IgM result (Table 3). Patients could be divided into 2 groups: those with a known reason (other than INKV or CHATV infection) for seeking medical care (10/17) and those for whom the cause of acute infection was unknown (7/17) (Table 3). The latter group of patients were evaluated for California serogroup virus infection because their medical records showed no other cause for their symptoms (i.e., no underlying disease or laboratory findings that implied another infection) (Table 4). All 4 children (≤16 years of age) with an unknown infection had

Table 2. Age group and sex of patients whose serum samples were tested and number of samples that were IgM positive for INKV and CHATV infections in Finland, 2001–2013*

Characteristic	Patients, no. (%)	INKV infection	CHATV infection
Age range			
0–9	670 (8.42)	1	0
10–19	717 (9.01)	2	0
20–29	905 (11.37)	0	0
30–39	1062 (13.34)	1	0
40–49	1180 (14.82)	2	1
50–59	1377 (17.30)	5	1
60–69	1093 (13.73)	1	0
>70	957 (12.02)	0	1
Sex			
F	3802 (47. 76)	8	0
M	4159 (52.24)	6	3

*CHATV, Chatanga virus; INKV, Inkoo virus.

Table 3. Clinical concurrent conditions and immunofluorescence and neutralization titers of patients with IgM-positive California serogroup virus infections in Finland, 2001–2013*

Patient no.	IgG IFA titer	IgM IFA titer	PCR result	Underlying illness	Reason for medical care	PRNT titer		
						INKV	CHATV	TAHV
1	160	160	Neg	–	Hospitalized (unknown infection)†	320	40	40
2	80	40	Neg	–	Hospitalized (unknown infection)†	320	640‡	40
3	120	40	Neg	–	Hospitalized (unknown infection)†	320	40	40
4	>640	>320	Neg	Hypertension	Hospitalized (unknown infection)†	320	20,480	5,120
5§	160	+/ND¶	ND	–	Hospitalized (unknown infection)†	ND	ND	ND
6§	320	+/ND¶	ND	–	Hospitalized (unknown infection)†	ND	ND	ND
7	80	>320	Neg	Type 2 diabetes, hypertension	Fever (unknown infection)†	>640	40	80
8	320	40	Neg	–	No information	320	<20	<20
9	80	80	Neg	Asthma, immunodeficiency	No information	160	40	<40
10	40	20	ND	Hypothyroidism	Follow-up visit (suspected MS, neurologic disorder)	160	20	40
11	320	>320	Neg	–	Follow-up visit (recurrent respiratory tract infections for 4 mo, suspected immunodeficiency)	320	40	80
12	<20	40	Neg	Schizophrenia, hypothyroidism	Follow-up visit (HSV eye infection, rash, Steven-Johnson syndrome)	320	20	40
13	160	120	Neg	–	Hospitalized, acute infection (*E. coli* urosepsis)	160	40	40
14	<20	120	Neg	MS disease, hypothyroidism	Hospitalized, acute infection (HSV infection)	>640	<20	20
15	320	120	Neg	–	Hospitalized, acute infection (impetigo contagiosa)	640	40	40
16	960	40	ND	–	Hospitalized, multiple infarcts in the central nervous system	1,280	5,120	1,280
17	80	30	Neg	–	Hospitalized, epidemic nephropathy	320	<20	<20

*CHATV, Chatanga virus; HSV, herpes viruses; IFA, indirect immunofluorescence; INKV, Inkoo virus; MS, multiple sclerosis; ND, PCR not done; PRNT, plaque reduction neutralization test; TAHV, Tahyna virus; –, no underlying illness.
†Full patient history describing INKV or CHATV infection.
‡4-fold difference between titers was not achieved with neutralization test, the diagnostic criterion used to confirm CHATV infection.
§INKV infection confirmed by hemagglutination inhibition test and neutralization test earlier in Helsinki University Central Hospital laboratory.
¶Samples tested were IgM positive, but titer was not tested.

acute INKV infection, whereas 2 adults with an unidentified infection had CHATV infection. These 6 patients were hospitalized. All had fever and other symptoms such as sore throat, nausea and vomiting, and neurologic conditions such as disorientation, nuchal rigidity, headache, and drowsiness. Small changes in electroencephalography were observed in 2 patients, and 1 patient had seizures (Tables 4, 5). All patients fully recovered from the infections.

Of the 17 California serogoup virus infection IgM-positive patients, 11 were not hospitalized for that infection. Four of these 11 patients visited a physician only once; 6 others were treated for another indication because their symptoms were interpreted as resulting from causes other than INKV or CHATV infection. Ten (91%) of the 11 patients who were not hospitalized were >40 years of age. Six (55%) of the 11 outpatients had a laboratory-confirmed co-infection with another pathogen, such as HSV (antigen positive), acute Puumala virus infection (i.e., the same serum sample was positive for Puumala virus IgM), an *Escherichia coli* urosepsis, and impetigo contagiosa. One patient was reported to have had erythema migrans 4 weeks before sample collection, yet no antibodies against *Borrelia burgdorferi* were found. One patient suffered a back injury 1 week before symptom onset (Table 3).

Discussion

In previous studies, the prevalence of California serogroup virus antibodies was high (30%–40%) in Nordic countries (*5,8*). To maintain such a high seroprevalence in Finland, >20,000 acute infections would need to occur annually during the mosquito season. Worldwide, California serogroup viruses other than INKV and CHATV have been associated with febrile illnesses and neurologic infection, but these infections have not been characterized in Finland. For that purpose, we retrospectively screened panels of serum samples that were originally collected over a period of years during the mosquito season and sent to our diagnostic laboratory for detection of antibodies to either causative agents of CNS infection (HSV1, HSV2, human herpesvirus 6, varicella zoster virus, *Mycoplasma pneumoniae*), Puumala virus, or INKV.

We estimated the frequency of acute human CHATV and INKV infections and characterized symptoms of these infections. The 2 patients with confirmed CHATV infection show that CHATV can cause human infection. We found symptoms that were similar to those reported for other viruses in the California serogroup. Both INKV and CHATV have remained practically unknown among physicians in Finland, and the rate of clinical suspicion has been

Table 4. Clinical progression of illness for patients hospitalized with acute INKV (n = 4) or CHATV infection (n = 2), Finland*

Virus and patient no.	Illness progression	Additional findings
INKV		
1	Day 1: fever 38°C, influenza-like symptoms Day 3: disoriented Day 6: hospitalized, abnormal EEG, CAL IgM+ Day 7: psychotic but discharged Day 10: follow-up EEG shows same abnormalities 3 mo later: EEG almost normal	Elevated HHV-6 antibody levels from same sample
2	Day 1: fever 39.5°C, headache, nuchal rigidity, sore throat before fever, hospitalized Day 2: nuchal rigidity, headache deteriorating, slowness but oriented Day 3: discharged, CAL IgM+ Day 5: headache again, hospitalized Day 6: discharged	Tick bite 1 mo earlier, erythema migrans; day 1: BorrAb neg
3	Day 1: vomiting Day 2: stomach pain, diarrhea, seizures, hospitalized Day 3: fever 38.3°C, drowsiness, convulsions Day 4: More seizures, small changes in EEG Day 5: CAL IgM+ Day 8: discharged	
4	Day 1: fever 37.9°C, sore throat Day 3: CAL IgM+ Day 4: nausea and vomiting Day 5: fever 39°C, headache, nuchal rigidity, hospitalized Day 10: recovered and discharged	Tick bite 3 wks earlier
CHATV		
1	Day 1: vomiting continuing for 3 d Day 4: fever, hospitalized, disoriented at night Day 7: frontal headache, normal head CT and abdominal ultrasound Day 12: discharged, CAL IgM+	
2	Day 1: fever 39°C, back pain Day 7: hospitalized, high fever, back pain almost resolved Day 7–22: temporal pain, trembling of hands, fluctuating fever Day 17: CAL IgM+ Day 23: discharged	Back injury 2 wks earlier

*BorrAb, Borrelia antibody test; CHATV, Chatanga virus; CT, computer tomographic scan; EEG, electroencephalogram; HHV, human herpesvirus; INKV, Inkoo virus. CAL IgM+ indicates the day when IgM for California encephalitis group viruses was observed.

negligible here (Table 1). The lack of awareness regarding these infections is similar to the situation with California serogroup viruses in many other countries.

A few articles from Russia have described the outcome and neurologic characteristics of INKV infection (*10*–*12*). In these studies, most patients had fever; almost 30% had neurologic symptoms (*11*). Studies in the Ryazan area showed that INKV infection was most frequently found in adults 21–40 years of age, and the number of cases peaked during the 2 periods of May and early August (*31*). In our study, 88% of the INKV cases were found during August and September or even later; only 2 cases occurred in early summer, and most patients with acute infection were ≤16 or 50–59 years of age. Another study from Russia included 520 selected patients; overall, 9.8% had California serogroup infection, with 2.5% and 1.2% INKV incidence in febrile and encephalitis patients, respectively (*11*). A more detailed study on the symptoms of the California serogroup infection included 118 patients, but INKV was confirmed in only 2 patients, 1 with multiple sclerosis and the other with meningoencephalitis (*10*). In that study, TAHV and undefined California serogroup infections (i.e., neutralization tests found no difference between INKV and TAHV) occurred more frequently than INKV (*10*). However, during 1995, the study period, CHATV had not yet been isolated but, as we now know, was already circulating in the area (*14*). Consequently, these infections could have been caused by CHATV.

Our study confirmed both INKV and CHATV infections in Finland. Most identified acute cases were from the Helsinki hospital district (Figure), possibly because most samples were collected in southern Finland, where the laboratory is located and where Finland's population density is highest. The overrepresentation of samples from the southern region may have decreased the likelihood of finding acute cases because distribution of California serogroup virus seroprevalence among humans is greater in northern Finland than in southern Finland, although the seroprevalence has recently increased in southern parts of the country (*5,32*). Furthermore, the high seroprevalence

Table 5. Symptoms of acute INKV and CHATV infections, as recorded in charts of 7 patients hospitalized with unknown infection, Finland*

Symptom	INKV, N = 5	CHATV, N = 2
Fever	5	2
Influenza-like symptom	4	0
Headache	4	2
Nausea/vomiting	2	1
Disorientation	2	1
Sore throat	2	0
Nuchal rigidity	2	0
Changes in EEG	2	0
Diarrhea	1	0
Seizure	1	0
Drowsiness	1	0

*These 7 patients were hospitalized with unknown infection (Table 3), which were determined to be Inkoo virus (INKV) or Chatanga (CHATV) infections. Symptoms are listed in order of frequency. EEG, electroencephalogram.

suggests that the serogroup viruses are found abundantly in nature and that infection is fairly common, so the frequency of acute cases we observed may underestimate the actual number of cases in Finland. Most acute cases occurred in autumn, yet the population density of the INKV principal vector, *O. communis*, is highest in June. Further studies are needed to determine whether this time lag reflects a long incubation period, spillover to other vectors, replication cycle in amplification hosts, or a change in vector occurrence.

The high seroprevalence in Finland with the low frequency of cases requiring healthcare and low frequency of diagnostic sampling suggests that most of these infections are subclinical or manifest as mild disease. On the other hand, the patient panels used in our study were highly selected, so we could have missed INKV and CHATV cases among patients with other symptom patterns. In addition, the IgM IFA test used may not have been sensitive enough to detect IgM in all cases. The high seroprevalence in the populations in Finland may also be influenced by the newly identified California serogroup virus isolate found here, the Chatanga virus Möhkö strain (*13*). Results from the diagnostic test in use show cross-reactions between the serogroup viruses. Most (82%) of the acute infections in this study were INKV cases, confirmed by using PRNT, which showed >4-fold titer differences, consistent with findings that INKV is the major California serogroup agent in seroprevalence studies in Finland and Sweden (*33*). Although most cases seem subclinical or mild, the data in our study indicate that the clinical disease may occasionally be severe; all children with acute illness had INKV infection and were hospitalized. Although INKV infection in adults was mild, 1 adult patient with CHATV infection required hospital care.

The considerable variation in IgG and IgM titers of serum samples from patients with acute infection may suggest that the samples were taken at different stages of the acute infection, but the differences may simply reflect variation in the antibody levels in individual patients. IgM was detectable in several patients concomitantly with high titers of IgG and neutralizing antibodies, a finding suggesting that IgM may persist for several weeks. One patient had detectable IgM 17 days after symptom onset (Table 4). A follow-up sample would be needed to show seroconversion and confirm the acute infection. No patient samples were collected during the viremic stage, and all samples

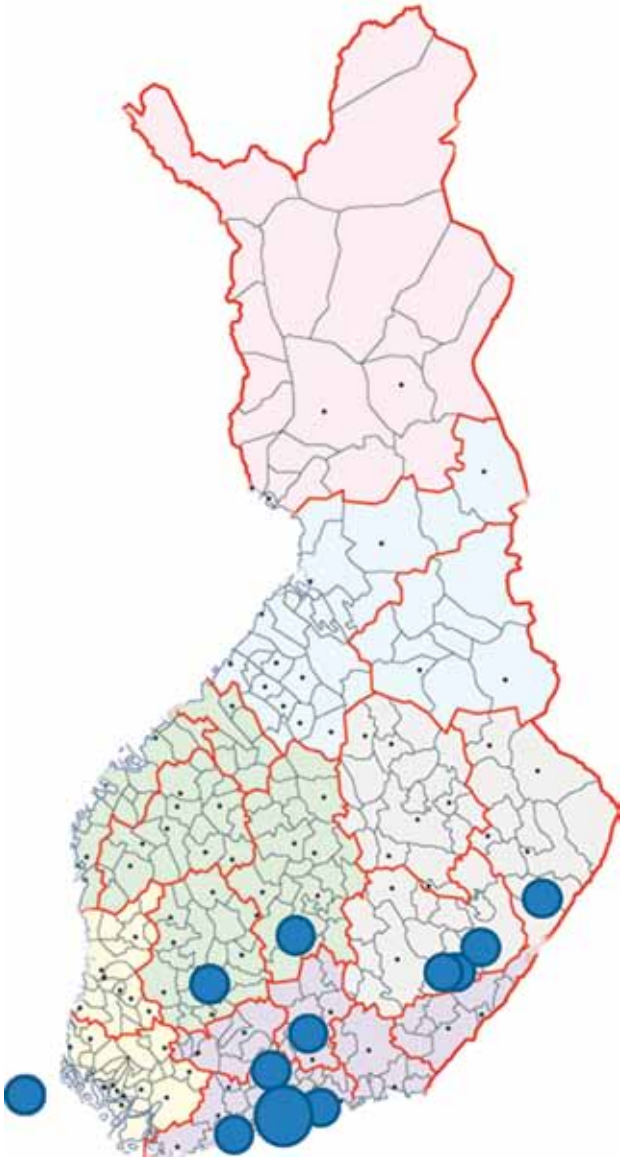

Figure. Locations of residence for 17 patients who were IgM positive for California serogroup virus infections, Finland. Each dot represents 1 patient except for the largest dot in southern Finland, which indicates a site for 6 patients. The dot on the far left indicates a patient from Åland Islands, Finland. Map source: National Land Survey of Finland (© 2015). A color version of this figure is available online (http://wwwnc.cdc.gov/EID/article/22/5/15-1015-F.htm).

were RT-PCR negative. Distinct LACV strains are known to cause different symptoms (*34*), but neither INKV nor CHATV have been isolated from a human sample. Human isolates or sequences of these viruses would be valuable for comparing the pathogenicity of the strains and analyzing cases in patients requiring hospitalization.

Many of our cases appear to represent recent subclinical infections that were identified only because of IgM testing and were unrelated to the reason for patients' visits to the healthcare unit. In the 2 patients with HSV cold sores, subclinical INKV infection could have triggered reactivation of HSV. Alternatively, INKV may require the presence of an underlying disease or trauma to cause a symptomatic infection (e.g., by enabling the virus to cross the blood–brain barrier). Some reports from Russia suggest that certain concomitant microbial infections may render the course of the INKV infection more severe (*11*).

In conclusion, we describe INKV and CHATV infections in humans and the clinical characteristics of acute disease. Symptoms of acute INKV and CHATV infections in patients in our study resembled symptoms of other California serogroup virus infections: influenza-like illness, with fever being most prominent. Most acute cases appeared to be subclinical, and a small minority of patients required hospitalization. Compared with adults, children were at higher risk for contracting more severe disease and were more often hospitalized because of INKV infection. In adults, CHATV infection appeared to be more severe than INKV infection. Further studies are required to explore in detail the clinical picture, prognosis, incubation period, and antibody kinetics of these infections. Viral isolates or RT-PCR–positive samples from patients are needed to acquire data related to INKV and CHATV strains causing the clinical cases.

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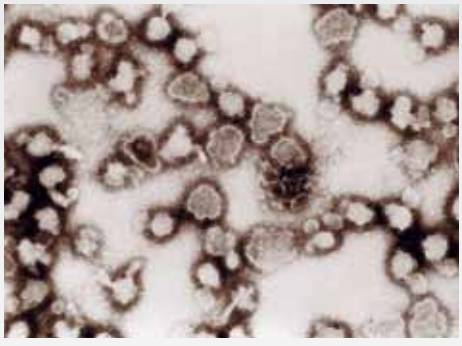
etymologia

Orthobunyavirus [or"tho-bun'yə-vi"rəs]

The largest genus in the family *Bunyaviridae*, the genus *Orthobunyavirus* was originally named *Bunyavirus*, for the type species Bunyamwera virus, first isolated in 1943 from the eponymous town in western Uganda. Originally, the vernacular term "bunyavirus" was used for viruses in this genus, but as more genera were added to *Bunyaviridae* (there are currently 5), confusion arose over whether "bunyavirus" referred to members of the genus *Bunyavirus* or family *Bunyaviridae*.

In 1995, the Bunyaviridae Study Group of the International Committee on Taxonomy of Viruses recommended adding the prefix "ortho-" (Greek for "correct") to the genus name (C. Calisher, pers. comm.) to prevent confusion. Two orthobunyaviruses reported on in this issue of *Emerging Infectious Diseases* are Inkoo virus and Chatanga virus (named for the towns of Inkoo, Finland, and Khatanga, Russia, respectively, where they were first isolated).



This electron micrograph reveals the morphologic traits of the La Cross virus (LCV), a Bunyaviridae virus family member. Photo: Public Health Image Library

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Differences in Genotype, Clinical Features, and Inflammatory Potential of *Borrelia burgdorferi* sensu stricto Strains from Europe and the United States

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Borrelia burgdorferi sensu stricto isolates from patients with erythema migrans in Europe and the United States were compared by genotype, clinical features of infection, and inflammatory potential. Analysis of outer surface protein C and multilocus sequence typing showed that strains from these 2 regions represent distinct genotypes. Clinical features of infection with *B. burgdorferi* in Slovenia were similar to infection with *B. afzelii* or *B. garinii*, the other 2 *Borrelia* spp. that cause disease in Europe, whereas *B. burgdorferi* strains from the United States were associated with more severe disease. Moreover, *B. burgdorferi* strains from the United States induced peripheral blood mononuclear cells to secrete higher levels of cytokines and chemokines associated with innate and Th1-adaptive immune responses, whereas strains from Europe induced greater Th17-associated responses. Thus, strains of the same *B. burgdorferi* species from Europe and the United States represent distinct clonal lineages that vary in virulence and inflammatory potential.

Incidence of Lyme borreliosis, the most common vectorborne disease in the Northern Hemisphere, is increasing. This disease usually begins with an expanding skin lesion, erythema migrans, which is often accompanied by nonspecific symptoms, such as headache, fatigue, myalgias, and arthralgias. If not treated, this infection can disseminate to the nervous system, heart, or joints (*1,2*).

Lyme borreliosis is caused primarily by 3 species of the *Borrelia burgdorferi* sensu lato complex: *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto (hereafter referred to as *B. burgdorferi*) (*3*). Variations in geographic distribution and clinical manifestations of this disease have been noted for each species. In Europe, infection is predominantly with *B. afzelii,* which usually remains localized to the skin, and *B. garinii*, which is usually associated with nervous system involvement (*1*). *B. burgdorferi* infection is rare in Europe; little is known about its clinical course there. In the United States, *B. burgdorferi* is the sole agent of Lyme borreliosis; in the northeastern United States, it is particularly arthritogenic (*1,2*). For all 3 species, the first sign of infection is often an erythema migrans lesion. However, *B. burgdorferi* infection in the United States is associated with a greater number of disease-associated symptoms and more frequent hematogenous dissemination than *B. afzelii* or *B. garinii* infection in Europe (*4–7*).

Clinical manifestations of Lyme borreliosis are believed to result from host immune response to the spirochete. Erythema migrans lesions of *B. burgdorferi*–infected patients in the United States have higher levels of mRNA for cytokines and chemokines associated with innate and Th1-adaptive immune responses than lesions from patients in Austria infected with *B. afzelii* (*4*). *B. burgdorferi*–infected patients in the United States also have higher levels of cytokines and chemokines in serum, and isolates from these patients induce macrophages to secrete more interleukin-6 (IL-6), IL-8, IL-10, chemokine ligand 3 (CCL3), CCL4, and tumor necrosis factor (TNF) than *B. afzelii* or *B. garinii* from patients in Slovenia (*8*).

These differences in inflammatory potential between *B. burgdorferi* from the United States and *B. afzelii* and *B. garinii* from Europe might account, in part, for differences in clinical manifestations of Lyme borreliosis. However, little is known about virulence and inflammatory capacity of *B. burgdorferi* in Europe, or how it compares with *B. burgdorferi* in the United States. In this study, we compared infection with *B. burgdorferi* in Europe and the United States by genotype, clinical manifestations, and inflammatory potential.

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Methods

Patients and Strains

Twenty-nine *B. burgdorferi* isolates were cultured from Lyme borreliosis patients in Slovenia (Central Europe) at the Institute of Microbiology and Immunology (Ljubljana, Slovenia). Isolates were identified as *B. burgdorferi* by using *Mlu*I large restriction fragment patterns (*9*). Twenty-four isolates were obtained from skin (19 from erythema migrans, 4 from acrodermatitis chronica atrophicans, 1 from borrelial lymphocytoma), and 5 from cerebrospinal fluid of patients with Lyme neuroborreliosis. These 29 strains represent all available patient-derived *B. burgdorferi* isolates collected during a 20-year period (1994–2013) at the Institute of Microbiology and Immunology. Clinical information and demographic data were obtained for same 29 patients at the Lyme Borreliosis Outpatient Clinic at the University Medical Center Ljubljana. The study was approved by Slovenian National Medical Ethics Committee (no.133/06/13).

In a study of patients with Lyme borreliosis in the United States (Rhode Island and Connecticut), 91 *B. burgdorferi* isolates were cultured from erythema migrans lesions (*10*). One isolate had a mixed genotype and was not included in our study. All patients met the Centers for Disease Control and Prevention (Atlanta, GA, USA) criteria for erythema migrans. The Human Investigation Committee at Tufts Medical Center and Massachusetts General Hospital approved this study.

Characterization of Strains

We genotyped *Borrelia* strains by using outer surface protein C (OspC), ribosomal RNA intergenic spacer type (RST), and multilocus sequence typing (MLST). OspC type was determined by using seminested PCR and sequencing. RST was determined by using nested PCR and restriction fragment length polymorphism analysis (*11,12*). OspC and RST genotypes were determined for all 29 *B. burgdorferi* isolates from Slovenia and 90 from the United States.

For MLST analysis, we amplified 8 chromosomal housekeeping genes (*clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*) by using nested PCR, sequenced in both directions (*13*), and analyzed by using CLC Main Workbench (http://www.clcbio.com/products/clc-main-workbench/). MLST analysis included 29 isolates from Slovenia and a representative subset of 41 isolates comprising the most common *B. burgdorferi* subtypes from the northeastern United States. Because of the prevalence of OspC type B among isolates from Slovenia, this analysis also included all 11 OspC type B isolates from the United States in our collection. New sequence types were submitted to the MLST database (http://pubmlst.org/borrelia/).

Phylogenetic Analysis

We constructed a minimum spanning tree by using BioNumerics version 7.1 software (Applied Maths, Austin, TX, USA). Comparison of strains from different regions included MLSTs of human *B. burgdorferi* isolates in the *Borrelia* MLST database. Phylogenetic trees of concatenated sequences of housekeeping genes were constructed by using MrBayes software (*14*).

Comparison of Clinical Findings

Demographic and clinical findings were available for 14 of 19 erythema migrans patients with *B. burgdorferi* infection from Slovenia and 90 erythema migrans patients with *B. burgdorferi* infection from the northeastern United States. We compared findings for *B. burgdorferi* infection with findings for 200 patients in Slovenia with *B. afzelii* infection and 116 with *B. garinii* infection; all had culture-positive erythema migrans.

Inflammatory Potential of *B. burgdorferi* Isolates

We assessed the inflammatory capacity of *B. burgdorferi* strains by stimulating peripheral blood mononuclear cells (PBMC) with 29 *B. burgdorferi* isolates and determining levels of cytokines and chemokines in culture supernatants. These isolates included 14 *B. burgdorferi* isolates from erythema migrans lesions of patients from Slovenia for whom detailed clinical information was available and 15 representative *B. burgdorferi* isolates (5 each of RST1, RST2, and RST3) from patients in the United States. For cell culture experiments, 29 low-passage (≤5) isolates were grown to mid-to-late log phase in complete Barbour-Stoenner-Kelly medium (Sigma-Aldrich, St. Louis, MO, USA) (*15*). Numbers of spirochetes in each culture were determined by optical density using a standard curve (*8*).

Human PBMC were obtained from 4 healthy donors at the Massachusetts General Hospital Blood-Component Laboratory, and PBMC were isolated from leukopaks by centrifugation in lymphocyte separation medium (MP Biomedicals, Santa Ana, CA, USA). Cells were cultured overnight in RPMI 1640 medium containing 10% human serum in 96-well plates (2×10^5 cells/well) at 37°C in 5% CO_2. To keep host factors constant, we stimulated PBMC from each healthy donor with each of 29 patient-derived *B. burgdorferi* isolates (multiplicity of infection = 25) in 4 independent experiments for 7 days (*16*).

We assessed protein levels of 22 cytokines and chemokines associated with innate and adaptive immune responses (innate: TNF, IL-1β, IL-6, IL-10, granulocyte–macrophage colony-stimulating factor, IL-8, IFN-α, CCL2, CCL3, and CCL19; adaptive-Th1: IFN-γ, IL-12p40, IL-12p70, cysteine-X-cysteine motif cytokine ligand 9 (CXCL9), and CXCL10; adaptive-Th17: IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, and IL-27) in culture supernatants by using

bead-based Luminex (EMD-Millipore, Darmstadt, Germany) multiplex assays. We averaged results from 4 experiments for analysis.

Statistical Analysis

We assessed differences between groups by using the Mann-Whitney rank-sum test and differences for categorical data by using the Fisher exact test (SigmaPlot-12.5; http://www.sigmaplot.com/products/sigmaplot/produpdates/prod-updates18.php0). p values <0.05 were considered significant.

Results

B. burgdorferi Genotypes

To examine genotypic characteristics of *B. burgdorferi* from Europe and the United States, we assessed 29 patient-derived isolates from Slovenia and 90 isolates from the United States by using the 2 most common typing systems, RST and OspC, which show strong linkage disequilibrium. Of 29 isolates from Slovenia, 21 (72%) were RST1 and 8 (28%) were RST3; none were RST2 (Figure 1; Table 1). Of 90 isolates from the United States, 38 (42%) were RST1, 39 (43%) were RST2, and 13 (14%) were RST3, a distribution consistent with those of other studies (*6,10,17,18*).

OspC typing showed that OspC type B (RST1) was the only OspC type found among isolates from Europe and the United States. Other OspC types were found exclusively in Slovenia (Q, R, L, S) or the United States (A, F, K, N, D, E, G, I). The most common *B. burgdorferi* strains in Slovenia were RST1-OspC type B (58%) and RST3-OspC type L (24%), whereas the most common strains in the northeastern United States were RST1-OspC type A (30%) and RST2-OspC type K (28%).

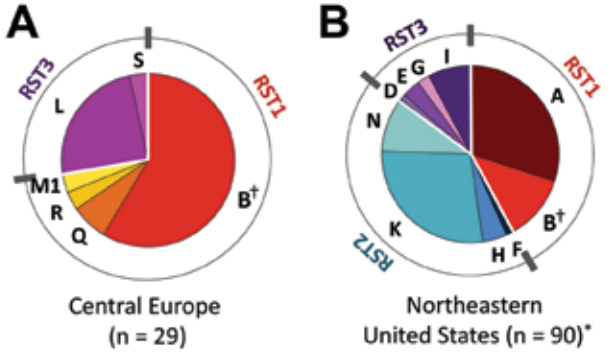

Figure 1. Distribution of *Borrelia burgdorferi* sensu stricto strains by outer surface protein C (OspC) and ribosomal RNA intergenic spacer type (RST). A) 29 isolates from patients with Lyme borreliosis in cental Europe (Slovenia). B) 90 isolates from patients with erythema migrans in the northeastern United States. OspC types are indicated by letters, and RSTs are indicated by colors. Red, RST1; blue, RST2; purple, RST3. *Based on previously published data (*10*). †Denotes OspC genotype (OspC type B) found in central Europe and the United States.

Table 1. Characterization of *Borrelia burgdorferi* sensu stricto strains, by RST and OspC, from patients in Slovenia and the United States*

Genotype	Slovenia, n = 29, no. (%)	United States, n = 90†, no. (%)
RST1	21 (72)	38 (42)
OspC type A	0	27 (30)
OspC type B‡	17 (58)	11 (12)
OspC type Q	2 (7)	0
OspC type R	1 (3)	0
OspC type M1	1 (3)	0
RST2	0	39 (43)
OspC type F	0	1 (1)
OspC type H	0	4 (4)
OspC type K	0	25 (28)
OspC type N	0	9 (10)
RST3	8 (28)	13 (14)
OspC type D	0	1 (1)
OspC type E	0	3 (3)
OspC type G	0	2 (2)
OspC type I	0	7 (8)
OspC type L	7 (24)	0
OspC type S	1 (3)	0

*RST, ribosomal RNA intergenic sequence type; OspC, outer surface protein C.
†Based on previously published data (*10*).
‡Found in central Europe and the United States.

MLST analysis (Table 2) included 29 isolates from Slovenia and 41 isolates from the United States, which comprised the major OspC types (A, K, and I) in the northeastern United States and all 11 OspC type B strains in our collection. Analysis identified 15 sequence types (STs). There was no overlap in STs from Europe and the United States, which demonstrated that strains of the same *Borrelia* species, including OspC type B strains, from the 2 regions were distinct genotypes.

Isolates from Slovenia represented 3 previously described STs and 2 new STs (ST545 and ST546) not in the MLST database. Strains from the United States represented a more heterogeneous group of 10 STs, all of which were reported previously in the MLST database. Three STs (ST9, ST20, and ST24) each comprised >1 OspC type. This result might be explained by greater propensity for horizontal transfer of genetic information at *ospC* gene loci, which are apparent recombination hotspots (*19*), whereas the 8 loci used in MLST analysis have lower rates of genetic recombination.

Minimum spanning tree and phylogenetic analyses of 70 *B. burgdorferi* strains underscored differences between strains in the 2 regions (Figure 2). Strains from Europe formed 2 clonal complexes (CC20 and CC24), which were separated by 3 and 6 alleles, respectively, from the most closely related strains in the United States.

Comparison of *B. burgdorferi* with *B. afzelii* or *B. garinii* (Figure 3) showed deeper branching among different *Borrelia* species but shallower branching within the same species, which demonstrated greater genetic discordance among species. Nevertheless, genotypic differences between *B. burgdorferi* in Europe and the

Table 2. Characterization of *Borrelia burgdorferi* sensu stricto strains, by MLST genotyping, from patients in Slovenia and the United States*

Genotype	No. (%) patients	
	Slovenia, n = 29	United States, n = 41†
RST1	21	21
OspC type A	0	10
MLST ST1	0	10 (24)
OspC type B	17	11
MLST ST20	6 (20)	0
MLST ST314	6 (20)	0
MLST ST545‡	5 (17)	0
MLST ST59	0	10 (24)
MLST ST7	0	1 (2)
OspC type Q	2	0
MLST ST20	1 (3)	0
MLST ST546‡	1 (3)	0
OspC type R	1	0
MLST ST20	1 (3)	0
OspC type M1	1	0
MLST ST20	1 (3)	0
RST2	0	10
OspC type F	0	1
MLST ST8	0	1 (2)
OspC type K	0	7
MLST ST3	0	6 (15)
MLST ST9	0	1 (2)
OspC type N	0	2
MLST ST9	0	2 (5)
RST3	8	10
OspC type D	0	1
MLST ST38	0	1 (2)
OspC type E	0	2
MLST ST19	0	2 (5)
OspC type G	0	2
MLST ST14	0	2 (5)
OspC type I	0	5
MLST ST16	0	5 (12)
OspC type L	7	0
MLST ST24	7 (24)	0
OspC type S	1	0
MLST ST24	1 (3)	0

*MLST, multilocus sequence typing; RST, ribosomal RNA intergenic sequence type; OspC, outer surface protein C; ST, sequence type.
†The 41 isolates from the United States were selected from a larger cohort that were representative of the most common RST/OspC subtypes in the northeastern United States. These isolates included all available OspC type B strains in our collection.
‡New ST.

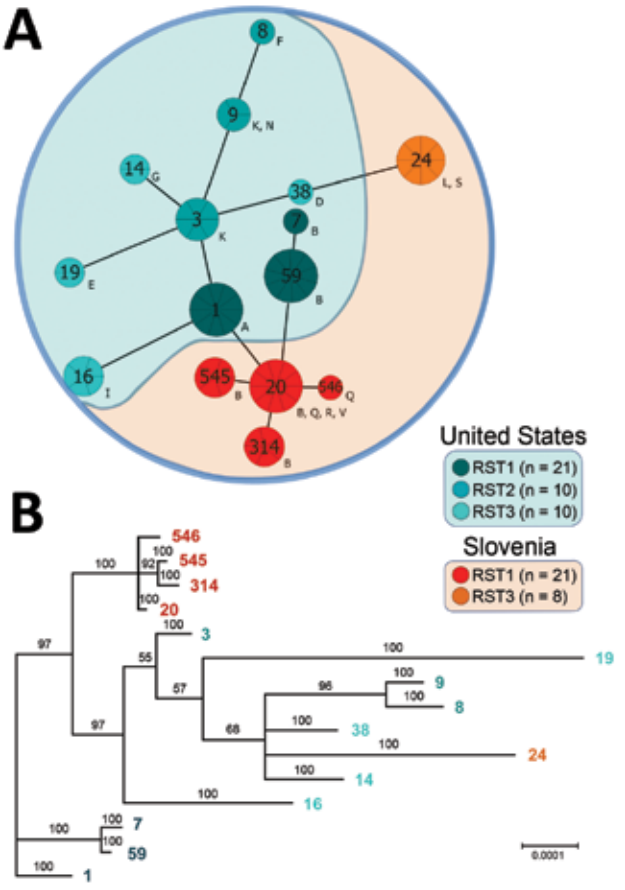

Figure 2. Phylogenetic analysis of *Borrelia burgdorferi* sensu stricto strains from central Europe (Slovenia) and the United States. A) Minimum spanning tree analysis of 70 isolates included in this study. Sequence types (STs) are indicated by numbers, and outer surface protein types are indicated by letters. Sizes of circles indicate ST sample sizes. Lengths of lines connecting STs indicate extent of variation (order of certainty) (no. locus variants). STs connected by the shortest black line are single-locus variants. Letters outside circles indicate OspC types. RST, ribosomal RNA intergenic spacer. B) Bayesian consensus tree resulting from simultaneous analysis of concatenated sequences of 8 housekeeping genes (*clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*). Values at nodes indicate Bayesian posterior probabilities (proportion of sampled trees containing the taxon bipartition). Scale bar indicates nucleotide substitutions per site.

United States, and closer clustering of strains within each geographic region, suggest divergence of *B. burgdorferi* on the 2 continents.

Clinical Findings for Patients with Erythema Migrans

We compared 14 *B. burgdorferi*–infected patients in Slovenia for whom detailed clinical information was available with 90 patients in the United States and found that patients from the United States had significantly shorter duration of erythema migrans at diagnosis (4 vs. 7 days; p = 0.02), greater frequency of associated symptoms (78% vs. 29%; p<0.001), and greater number of associated symptoms (4 vs. 0 symptoms; p = 0.005) (Table 3). *B. burgdorferi* from the United States differed from *B. afzelii* and *B. garinii* for most clinical parameters measured. In contrast, clinical features of *B. burgdorferi* infection in Slovenia were similar to those for *B. afzelii* or *B. garinii* infections (Table 3), despite substantial genotypic differences among species (Figure 3).

Differences between *Borrelia* species from Europe and the United States were most apparent for symptomology of infection (Figure 4). In the United States, erythema migrans is typically associated with fever, neck stiffness, malaise, and fatigue. However, these symptoms were substatially

less common in Slovenia. Thus, despite greater phylogenetic similarity with *B. burgdorferi* from the United States, infection with *B. burgdorferi* strains from Europe reflected more closely findings for 2 genetically discordant *Borrelia* species in Europe (*B. afzelii* and *B. garinii*) with which it shares an ecologic niche. In contrast, *B. burgdorferi* from the United States appeared to be more virulent.

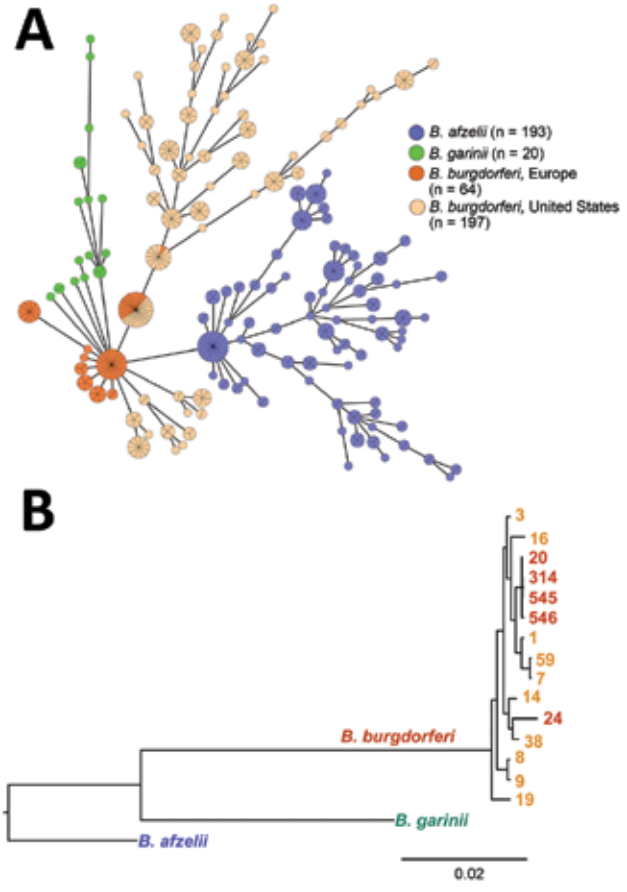

Figure 3. Phylogenetic comparison of 3 major pathogenic *Borrelia* species (*Borrelia afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto) that cause Lyme borreliosis. A) Minimum spanning tree analysis of 474 *B. burgdorferi* sensu lato human isolates. Analysis included 404 previously published datasets available in the multilocus sequence typing database (http://pubmlst.org/borrelia/) as of May 5, 2015, and 70 *B. burgdorferi* sensu stricto isolates from this study. Circles and numbers indicate specific sequence types (STs). Sizes of circles indicate MLST sample size and colors indicate origin of isolates. Lengths of lines connecting STs indicate order of certainty. STs connected by the shortest line are single locus-variants. B) Bayesian consensus tree resulting from simultaneous analysis of concatenated sequences of housekeeping genes of 70 *B. burgdorferi* sensu stricto isolates included in this study, representative strains of *B. afzelii* (http://pubmlst.org/borrelia/id:1546), and *B. garinii* (http://pubmlst.org/ borrelia/id:1829). Values at nodes indicate Bayesian posterior probabilities (proportion of sampled trees containing the taxon bipartition). Scale bar indicates nucleotide substitutions per site.

Inflammatory Responses in PBMC Stimulated with *B. burgdorferi*

To determine whether *B. burgdorferi* from the United States and Europe vary in inflammatory potential, we assessed levels of 22 cytokines and chemokines in supernatants of healthy human PBMC stimulated with 14 *B. burgdorferi* isolates from patients in Slovenia and 15 representative isolates from patients in the United States (5 each RST1, RST2, and RST3), for whom detailed clinical information was available. All 29 isolates were tested by using PBMC from each of 4 healthy donors, and results from 4 experiments were averaged for analysis.

Isolates from the United States and Europe induced greater expression of most cytokines and chemokines tested compared with unstimulated controls (Figure 5). However, *B. burgdorferi* from the United States induced higher levels of several mediators associated with innate immune responses, including IL-1β, IL-8, IL-10, TNF, and CCL3, than did *B. burgdorferi* from Europe (Figure 5, panel A). A similar trend was observed for Th1-associated mediators (IL-12p40, INF-γ, INF-γ–inducible CXCL9, and CXCL10), which are strong chemoattractants for CD4+/CD8+ T-effector cells (Figure 5, panel B). In contrast, levels of several Th17 mediators, including IL-17A, IL-22, and IL-27, were higher in cells stimulated with *B. burgdorferi* from Europe. Because immune response seems to be critical in disease expression, these differences in inflammatory responses might contribute to differences in clinical features of Lyme borreliosis in Europe and the United States.

Discussion

We compared infection with *B. burgdorferi* in Europe and the United States by genotype, clinical manifestations, and inflammatory potential. Strains in Europe differed from strains in the United States for all 3 parameters, which demonstrates transcontinental diversification of this species.

Although *B. burgdorferi* from Slovenia and the United States are depicted as the same species, they represent distinct clonal complexes that vary in capacity to induce host inflammatory immune responses and clinical features of disease. Clinical and immune characteristics of *B. burgdorferi* from Europe more closely resemble those of the phylogenetically distinct species *B. afzelii* and *B. garinii* from Europe, with which they share an ecological niche, than those of *B. burgdorferi* from the northeastern United States. These findings underscore divergence of *B. burgdorferi* strains on 2 continents. Moreover, data indicate a convergence of certain features among disparate *Borrelia* species within the same region, presumably through sharing of genetic information (*21*).

Three RST and >30 OspC genotypes have been identified in *B. burgdorferi* obtained from various sources (*11,12*), including 24 OspC subtypes that cause infection in

Table 3. Clinical characteristics of erythema migrans patients infected with *Borrelia afzelii*, *B. garinii*, or *B. burgdorferi* sensu stricto in Slovenia and *B. burgdorferi* sensu stricto in the United States*

Characteristic	Slovenia			United States,	
	B. afzelii, n = 200†	*B. garinii*, n = 116†	*B. burgdorferi* sensu stricto, n = 14	*B. burgdorferi* sensu stricto, n = 90†	p value‡
General					
Age, y	50 (15–79)	54 (20–83)	50 (3–77)	49 (15–82)	0.6
Female sex	128 (64)	61 (53)	10 (71)	44 (49)	0.2
EM duration at study entry, d	9 (1–206)	5 (1–61)	7 (2–53)	4 (1–30)	**0.02**
EM diameter, cm	13 (5–86)	18 (5–72)	12 (5–38)	11 (5–56)	0.6
Symptoms at study entry					
Symptoms/patient	0 (0–6)	0 (0–7)	0 (0–6)	4 (0–9)	**0.005**
Patients with symptoms	66 (33)	43 (37)	4 (29)	70 (78)	**<0.001**
Fatigue	51 (26)	23 (20)	3 (21)	55 (61)	**0.01**
Arthralgia	30 (15)	21 (18)	3 (21)	42 (47)	0.1
Myalgia	28 (14)	22 (19)	3 (21)	28 (32)	0.7
Headache	27 (14)	21 (18)	4 (29)	45 (50)	0.2
Fever, chills	12 (6)	10 (9)	2 (14)	41 (46)	**0.05**
Neck stiffness	9 (5)	4 (3)	0	43 (48)	**0.002**
Malaise	21 (11)	21 (18)	2 (14)	47 (52)	**0.01**

*Values are median (range) or no. (%). EM, erythema migrans.
†Based on previously published data: *B. burgdorferi sensu stricto* (*10*); *B. afzelii* (*5*); *B. garinii* (*20*).
‡For comparison of *B. burgdorferi* sensu stricto from Slovenia versus the United States. Values in bold indicate statistical significance.

humans (*22*). On the basis of 3 studies that evaluated genotypes of *B. burgdorferi* isolates that were obtained primarily from ticks from Europe or the United States (*13,22,23*), most OspC types were believed to be present exclusively in North America or Europe, whereas a few OspC types (A, B, K, E, L) were present in both continents (*22–24*).

Our study of *B. burgdorferi* from patients in Slovenia or the United States partially corroborates these findings and demonstrates that strains from these 2 regions are genetically distinct. However, of 15 OspC types identified, OspC type B was the only genotype found among isolates from both Slovenia and the United States. The high frequency of OspC type B (58%) in patients from Slovenia and absence of OspC types A, K, or E, which were reported in several countries in Europe (*13,22,23*), suggests that there might be regional variation in distribution of *B. burgdorferi* in different locations in Europe. Moreover, although not found in the northeastern United States, the high prevalence of OspC type L among patients in Slovenia and its recovery from patients in the midwestern United States (*24*) indicate that this strain also causes human disease.

Analysis of human *B. burgdorferi* isolates by MLST confirmed that strains on the 2 continents represent different clonal complexes, which is consistent with previous findings in isolates from ticks (*13,25*). STs of the 29 isolates from Slovenia differed from STs of the 157 isolates from North America in the MLST database, which implies transcontinental diversification of *B. burgdorferi*. Moreover, phylogenetic and minimum spanning tree analyses showed that isolates from Slovenia clustered in 2 distinct groups (ST20 and ST24), which suggests that strains in these groups evolved independently.

Although speculative, the data suggest that there might have been 2 independent divergence events for *B. burgdorferi* in Europe and the United States. One event

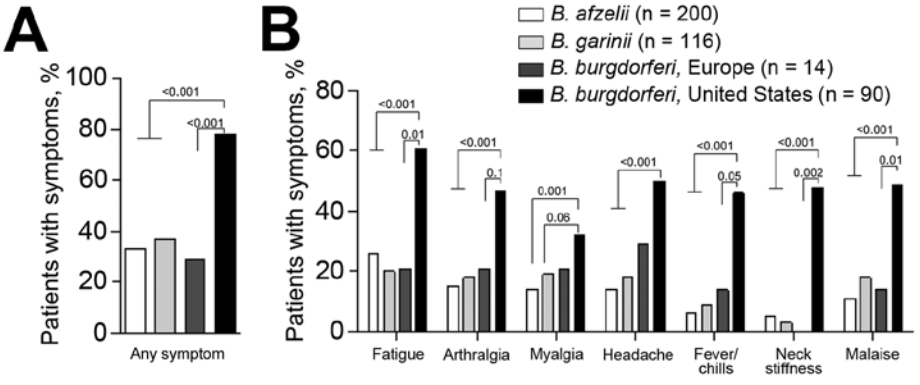

Figure 4. Frequency of symptoms in patients with erythema migrans infected with *Borrelia afzelii*, *B. garinii*, or *B. burgdorferi* sensu stricto from central Europe and *B. burgdorferi* sensu stricto from the United States. A) Any symptom, B) individual symptoms. Patients were assessed for 8 symptoms (fatigue, arthralgia, myalgia, headache, fever, chills, neck stiffness, or malaise). White bars indicate patients from Europe infected with *B. afzelii*, light grays bars indicate patients from Europe infected with *B. garinii*, dark gray bars indicate patients from Europe infected with *B. burgdorferi* sensu stricto, and black bars indicate patients from the United States infected with *B. burgdorferi* sensu stricto. Differences between strains were assessed by using the Fisher exact test. p values are indicated. There were no differences between *B. afzelii*, *B. garinii*, or *B. burgdorferi* sensu stricto from Slovenia.

involved migration of ST20, which is most closely related to RST1-OspC type A and B strains that are prevalent in the northeastern United States. A separate event involved ST24, which is closely related to OspC type L strains that were found in patients from the midwestern United States, but not in patients from the northeastern United States. However, these insights are based on a small sample size of isolates, all from humans. Genome analyses of larger numbers of isolates from various sources could provide better resolution of the geographic spread of *B. burgdorferi* (*19*).

Species determination is based on sequence homology of 16S rRNA, a highly conserved chromosomal region of the *Borrelia* genome that undergoes slow evolutionary change. However, ≈40% of borrelial DNA is located on plasmids, including highly polymorphic genes, such as *ospC* (*26*), which encode immunogenic proteins involved in spirochetal virulence. These genes are believed to be under considerable evolutionary pressure, and because of plasmid plasticity, there is evidence for gene recombination and lateral transfer of genetic information among strains (*21*). Thus, despite comprising the same species on the basis of 16S rRNA, *B. burgdorferi* from Europe and the United States appear divergent in expression of genes, such as *ospC*, that are responsible for immunogenicity and virulence. Consequently, strains defined as the same species might cause a spectrum of disease with different clinical features. Thus, species determination on the basis

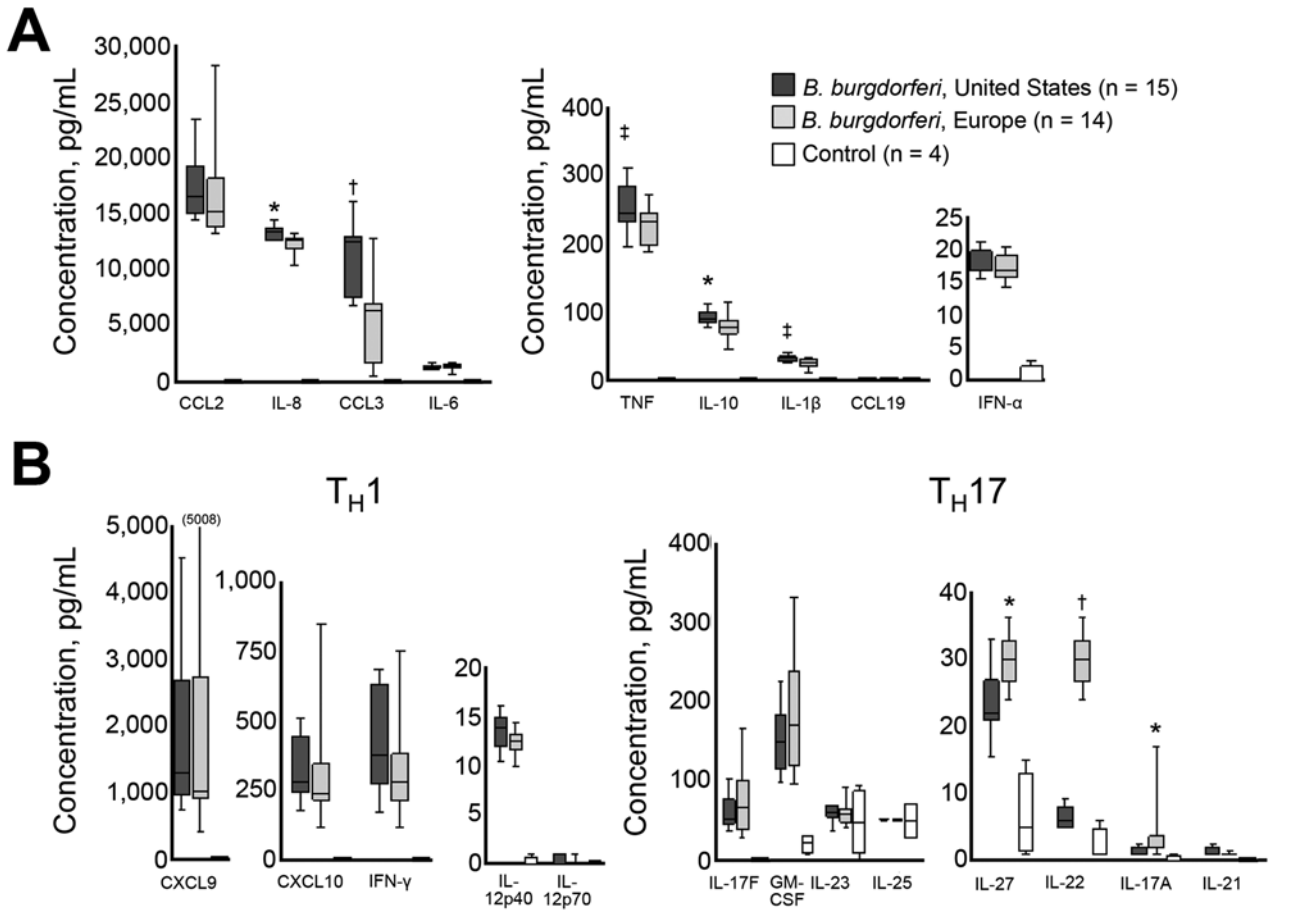

Figure 5. Inflammatory potential of *Borrelia burgdorferi* sensu stricto from Europe and the United States. Levels of 22 cytokines and chemokines associated with innate (A) or adaptive (B) immune responses. Innate responses: tumor necrosis factor (TNF), interleukin-1b (IL-1b), IL-6, IL-10, granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-8, chemokine ligand 2 (CCL2), CCL3, and CCL19. Adaptive immune responses: Th1: interferon-γ (IFN-γ), IFN-α, IL-12p40, IL-12p70, cysteine-X-cysteine motif chemokine ligand 9 (CXCL9), and CXCL10; Th17: IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, and IL-27. Immune responses were assessed in peripheral blood mononuclear cell culture supernatants after 7 days of stimulation with 14 *B. burgdorferi* sensu stricto isolates from Europe or 15 *B. burgdorferi* sensu stricto isolates from the United States (multiplicity of infection = 25) by using bead-based Luminex (EMD-Millipore, Darmstadt, Germany) multiplex assays. Each of 29 *B. burgdorferi* sensu stricto isolates was tested with peripheral blood mononuclear cells from each of 4 healthy donors in 4 independent experiments. Cytokine and chemokine values from the 4 experiments were averaged for analysis. Data are presented as box and whisker plots, boxes indicate interquartile ranges (1st and 3rd quartiles), lines inside boxes indicate median values, and error bars indicate 10th and 90th percentiles (value in parenthesis indicates the highest value). For comparison of *B. burgdorferi* sensu stricto isolates from Slovenia or the United States, *p≤0.01, †p≤0.001, ‡p≤0.05.

of genetically conserved regions of the genome might not adequately reflect the difference in virulence.

Researchers have reported differences in clinical features of infection with *B. garinii* and *B. afzelii* in Europe and *B. burgdorferi* in the northeastern United States (*4,5,20,27–30*). We extended these findings by showing differences in clinical features of erythema migrans caused by *B. burgdorferi* in Slovenia compared with *B. burgdorferi* in the United States. Despite substantial phylogenetic discordance among species, clinical features of infection with *B. burgdorferi* in Slovenia more closely resembled those of milder infections with *B. afzelii* and *B. garinii*, the 2 other *Borrelia* species that cause disease in Europe, than the more symptomatic infection associated with more closely phylogenetically related *B. burgdorferi* from the United States. These findings suggests sharing of genetic information among different *Borrelia* species.

Although we cannot exclude the possibility that host genetic or cultural differences might contribute to differences in clinical features of Lyme borreliosis in Slovenia and the United States, we do not believe that these differences are major factors. First, all study patients at both sites were of European descent and probably similar genetically. Second, evaluation of patients in both locations was similar and included assessment of objective measures, such as fever and erythema migrans diameter and duration, which would not be influenced by cultural differences in reporting symptoms. Third, median duration of erythema migrans in patients in the United States was likely shorter because these patients had more associated symptoms and sought treatment sooner than patients in Slovenia. In support of this interpretation, patients in Europe with *B. garinii* infection, which causes more pronounced itching and burning of erythema migrans lesions than other *Borrelia* species (*29*), had a similar duration of erythema migrans at study entry as patients in the United States. Thus, we believe that differences among *Borrelia* strains are the critical factor in explaining differences in clinical features of Lyme borreliosis on the 2 continents.

Our analysis in this study focused on clinical features of erythema migrans. However, distinctions in disease pathogenesis between *B. burgdorferi* strains from Slovenia and the United States are probably not limited to early disease. Of 29 *B. burgdorferi* isolates from Slovenia, 1 was obtained from a patient with borrelial lymphocytoma, and 4 were obtained from patients with acrodermatitis chronica atrophicans, a late disease manifestation. These clinical manifestations are rarely, if ever, seen in the northeastern United States, where late in the disease, *B. burgdorferi* is commonly associated with development of arthritis, which occurs rarely in Europe. These observations are consistent with a recent report suggesting higher frequency of Lyme neuroborreliosis after *B. burgdorferi* infection in Europe than in the United States (*25*). Thus, clinical differences between *B. burgdorferi* in Europe and the United States probably involve manifestations other than those associated with early infection.

Although there is a range of inflammatory potential for a given *Borrelia* RST strain (*31*), we previously showed in vivo and in vitro that *B. burgdorferi* from the northeastern United States induced greater inflammatory responses than *B. afzelii* and *B. garinii* from Europe (*4,8*). In this study, we demonstrated in cell culture that *B. burgdorferi* from Europe and the United States vary in inflammatory potential. Strains from the United States induced higher levels of cytokines and chemokines associated with innate immune responses and showed a similar trend for Th1-associated mediators. In contrast, strains from Europe induced higher levels of several Th17-associated cytokines.

The functional consequence of these differential immune responses is not known. Th17-associated mediators are detected in only a subset of patients with Lyme borreliosis and might not be as effective in spirochetal killing as innate or Th1-adaptive responses (*32*). However, because Lyme borreliosis patients are given antibiotic drugs, it is not known whether the natural history of the disease would be different in patients with predominantly Th1 or Th17 responses to the spirochete.

In addition to divergence of *B. burgdorferi* between Europe and the United States, emerging evidence suggests regional strain variation on each continent. A study of *B. burgdorferi* from humans in the midwestern and northeastern United States reported differences among isolates at these locations (*24*). In the northeastern United States, OspC types A (≈30%) and K (≈30%) are most common, whereas there is greater diversity in the midwestern United States, and OspC type H (≈20%) is most common. Although not assessed systematically, we believe that Lyme borreliosis is a generally milder disease in the midwestern United States. Similarly, the predominance of OspC type B and L strains in human isolates from Slovenia and absence of OspC type A and K strains found in other regions of Europe suggests region-specific diversification of strains. It will be useful to determine whether there are regional variations in Lyme borreliosis in Europe and Asia, where *B. afzelii* and *B. garinii* predominate. Greater knowledge of regional differences in infection might help clinicians in diagnosis and treatment specific for their region.

In conclusion, *B. burgdorferi* from Europe and the United States represent distinct genotypes that vary in inflammatory potential and clinical manifestations of Lyme borreliosis. Despite greater genetic discordance among *Borrelia* species, clinical features of *B. burgdorferi* infection in Europe appear similar to those for *B. afzelii* or *B. garinii* infection, the most prevalent *Borrelia* species in

Europe, indicating that strains within a particular regional environment, under similar evolutionary pressures, accrue similar characteristics as other strains that share the same ecologic niche.

K.S., F.S., and A.C.S. designed the study; T.C. and K.S. performed experiments and analyzed data; F.S., D.S., and A.C.S provided patient samples and clinical information; E.R.-S., A.C.S., and G.M. provided *Borrelia* isolates; E.R.-S., and G.M. provided assistance with culture and evaluation of isolates; and K.S., F.S., A.C.S., and T.C. wrote the article. All authors participated in developing, reviewing, and approving the article.

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Projecting Month of Birth for At-Risk Infants after Zika Virus Disease Outbreaks

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The marked increase in infants born with microcephaly in Brazil after a 2015 outbreak of Zika virus disease suggests an association between maternal Zika virus infection and congenital microcephaly. To project the timing of delivery of infants born to mothers infected during early pregnancy in 1 city in Bahia State, Brazil, we incorporated data on reported Zika cases and microcephaly cases into a graphical schematic of weekly birth cohorts. We projected that these births would occur through February 2016. Applying similar projections to a hypothetical location at which Zika virus transmission started in November, we projected that full-term infants at risk for Zika virus infection would be born during April–September 2016. We also developed a modifiable spreadsheet tool that public health officials and researchers can use for their countries to plan for deliveries of infants to women who were infected with Zika virus during different pregnancy trimesters.

In May 2015, the World Health Organization (WHO) reported an outbreak of Zika virus disease in Brazil (*1*). Zika virus is a single-stranded RNA virus spread primarily by *Aedes aegypti* mosquitoes; maternal–fetal transmission of Zika virus has been reported (*2*). Infection is asymptomatic in many patients; when clinical illness does occur, it is generally mild, with exanthematous rash, fever, conjunctivitis, or arthralgia (*3*). An association with Guillain-Barré syndrome is under investigation; on rare occasion, death of patients with chronic disease has been reported (*4*).

In October 2015, Brazil started to report higher than expected rates of microcephaly among infants born in the same states where Zika outbreaks had occurred several months before (*5*). Laboratory tests later confirmed Zika virus infection in several infants born with microcephaly, and several case series have reported that mothers who delivered an infant with microcephaly had experienced Zika symptoms during early pregnancy (*5*–*8*). Because of the potential link between Zika virus infection and microcephaly, on February 1, 2016, WHO declared a public health emergency of international concern (*9*,*10*).

As of February 26, 2016, WHO reported 31 countries and territories (*11*) in the Americas in which local vector-borne transmission of Zika virus was ongoing (*12*). With expanding local transmission and the possible link between infection during pregnancy and congenital microcephaly, projecting the effects of Zika virus infections for other countries and understanding the gestational time when risk is greatest are critical. As Zika virus has spread through the Americas, questions have arisen about the remarkably high numbers of infants with microcephaly reported in Brazil and the absence of reported microcephaly cases in some other countries where transmission is high. To help answer these questions, assessment of the timing of transmission and its relation to gestational week of pregnancy for the cohort of women who were pregnant during the outbreak is necessary. Our report illustrates the expected periods of exposure and weeks of delivery for the cohorts of pregnant women potentially infected with Zika virus during outbreaks in Bahia State, Brazil. Public health officials and researchers in areas with local transmission could apply these methods to country-specific data to produce more precise models and predictions.

Methods

Using published data for Bahia State and assuming that all pregnancies lasted 40 weeks (full term), we created figures demonstrating cohorts of pregnant women by week of delivery and then extrapolated to the beginning of pregnancy. Live-birth data from Brazil showed small differences in the proportions of infants born at full term (37–41 weeks) with microcephaly (76.7%) compared with those born at full term without birth defects (83.6%) (*13*). We considered the first 2 weeks of pregnancy to be the time from last menstrual period to conception (Figure 1). We also assumed the number of births to be constant across months of the year. To indicate the probable high-risk period for Zika virus transmission, we graphed the number of reported cases of Zika disease or Zika-like illness by epidemiologic week (the standardized method to enable comparison of weeks

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across years). We also graphed the reported cases of microcephaly by month of report, assuming that the month of report reflected the month of birth (*15*).

In Bahia, ≈4,000 infants are born each week (*16*); therefore, each bar represents ≈4,000 pregnancies. We derived epidemiologic data from a published report on exanthematous illness in the city of Salvador, Bahia State, Brazil (*14*). We assumed that the epidemic curve of exanthematous illness was representative of the epidemic curve of Zika virus infection and that the epidemic curve for the city of Salvador could be extrapolated to Bahia State. Because exact numbers of cases were not available, we derived estimates from the published epidemic curve, which was sufficient to identify the period of high Zika activity as being from March through June 2015. From the Live Birth Information System in Brazil (*16*), we obtained the monthly reports of infants born with microcephaly during August 2015–February 2016; information on births from January 2016 on were probably incomplete or were not yet available. The expected baseline prevalence of microcephaly is 6 cases per 10,000 births; for a state with 16,000 births per month, 10 cases of microcephaly would be expected each month.

To project the probable timing of births with adverse effects associated with Zika virus infection in early pregnancy, we then applied this approach to a hypothetical country. We assumed that transmission in Country A began on October 4, 2015, and followed the patterns that were seen in Salvador (*14*) and Yap Island (*3*). That is, we assumed that the level of transmission during October was low, during early November 2015 through mid-February 2016 was high, and from mid-February through mid-March 2016 was lower (Figure 2).

Results

In the city of Salvador, Zika virus transmission was highest during March–June 2015 (Figure 1) (*14*). During this period, a cohort of pregnant women could have been infected, and these infections would have occurred at different times during their pregnancies. The period of highest Zika activity was March 22–May 31, 2015 (Figure 1) across all cohorts. Pregnancies that began during November 2014–June 2015 correspond to births anticipated during August 2015–March 2016. For pregnancies that began in December 2014 or January 2015, the highest likelihood of Zika virus infection would have been late in the first trimester or during

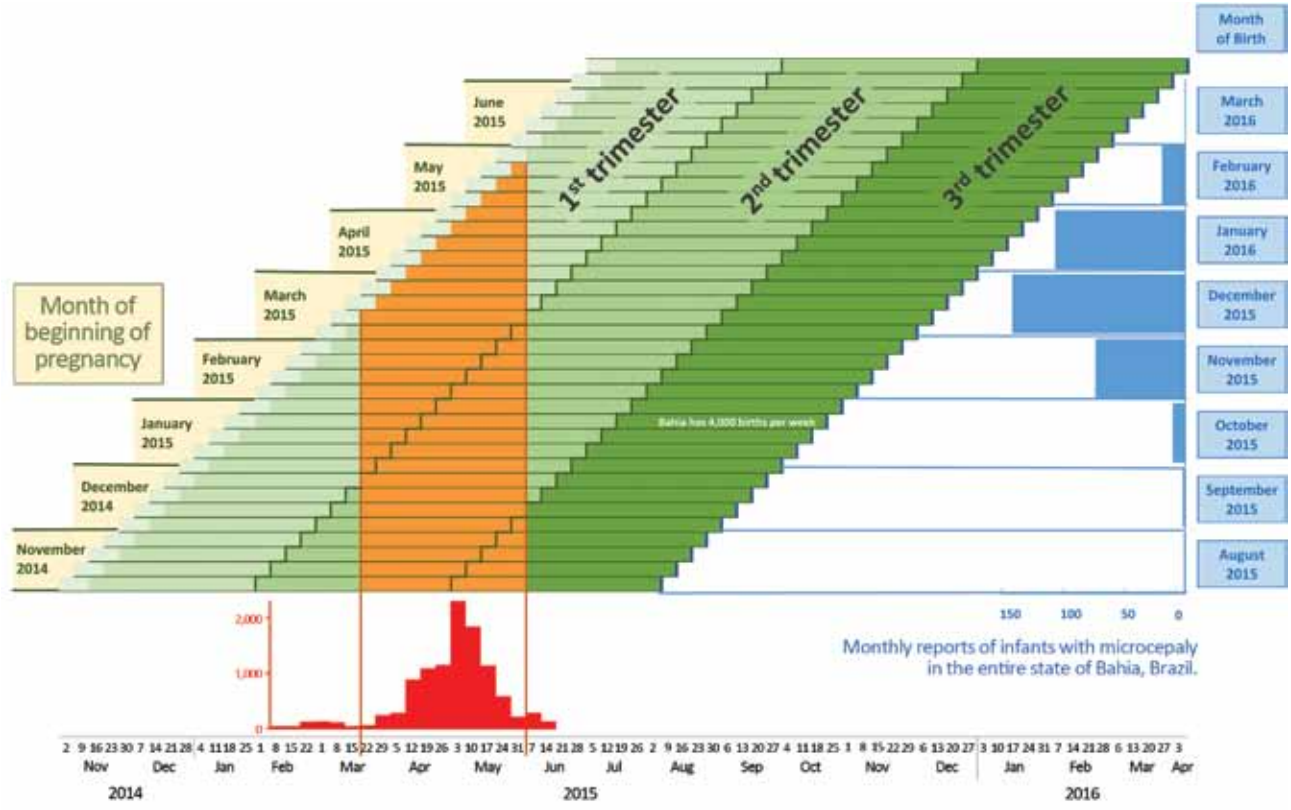

Figure 1. Projection of birth months after Zika virus transmission and occurrence of microcephaly, Salvador, Bahia State, Brazil. Weekly pregnancy cohorts are based on 40-week pregnancies and monthly reports of infants with microcephaly in Bahia State, Brazil, in relation to periods of high risk for Zika virus transmission. The epidemic curve shows cases treated for illness with rash in Salvador, Brazil, estimated from (*14*). Complete monthly report data for January–March 2016 are not yet available.

the second trimester of pregnancy, and these pregnancies would have resulted in term births during September and October 2015. For pregnancies that began during late February 2015–May 2015, the highest likelihood of Zika virus infection would have been during the first trimester, and term births would have occurred during November 2015–February 2016.

The increased number of reported cases of microcephaly in Bahia State began with October births; reported cases rose sharply during November 2015–January 2016. For the city of Salvador, these November 2015–January 2016 births corresponded to the highest likelihood of infection occurring in the first trimester or early in the second trimester of pregnancy, assuming that the date of report approximates the date of birth. There are no reports to indicate whether the city of Salvador experienced the Zika virus disease outbreak earlier or later than the rest of Bahia State.

In Country A (Figure 2), for the cohort of women whose pregnancies began in May 2015, corresponding to births during February–early March 2016, the likelihood of Zika virus infection would have been limited to the third trimester of pregnancy. Women whose pregnancies began in July 2015 would be expected to deliver in late March and early April 2016, and risk for infection would have been highest during the second trimester. The highest likelihood of first trimester and early second trimester infection would be among women who became pregnant during September 2015–January 2016, which corresponds to births from mid-May through early October 2016.

To enable readers to project months when births with exposure in different trimesters can be expected, we developed a modifiable spreadsheet tool (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/5/16-0290-Techapp1.xlsx). Users may enter start and end dates of hypothetical outbreaks.

Discussion

Our projections, based on ecologic data, indicate that in Bahia State, Brazil, Zika virus infection during the first trimester or early in the second trimester of pregnancy is temporally associated with the observed increase in infants born with microcephaly; this projection is consistent with the observed

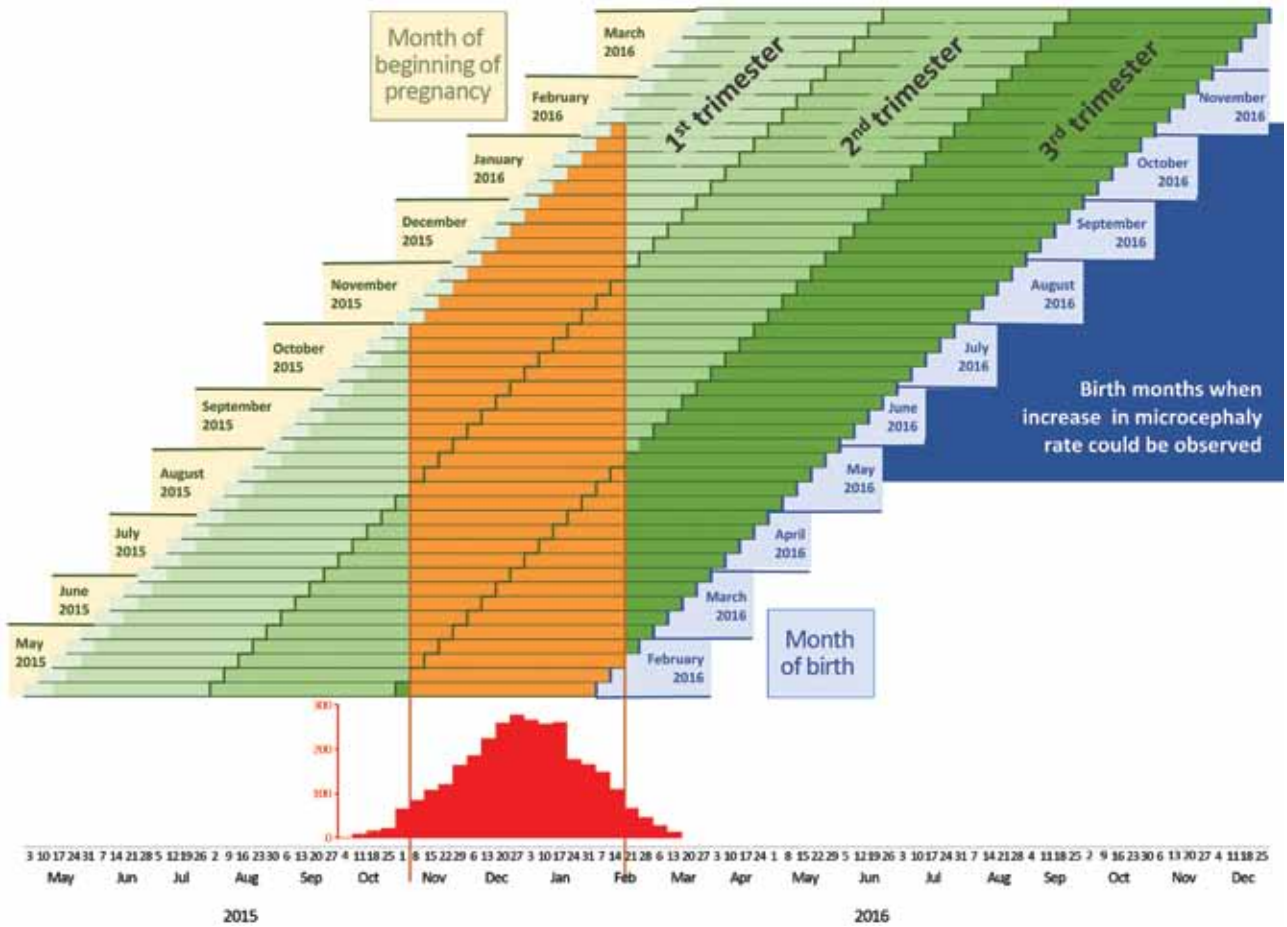

Figure 2. Projection of anticipated birth months after Zika virus transmission in a hypothetical country. Projected birth months for weekly pregnancy cohorts are based on 40-week pregnancies in a hypothetical country in which the highest level of Zika activity was from November 2015 through mid-February 2016.

reported decline for January and February 2016. This finding adds to pathologic findings documenting Zika virus infection in several infants with microcephaly (*7*,*8*). To create a more precise projection of when to expect the first full-term births to mothers who were infected during their second trimester of pregnancy, readers can refine our model by using our modified spreadsheet tool (online Technical Appendix) and local data from countries in which Zika virus is transmitted.

Understanding the timing of Zika virus infection of pregnant women is key because the effects of infection on pregnancy and fetal and infant outcomes is likely to vary by gestational timing, as has been demonstrated for other congenital infections such as rubella and cytomegalovirus; transmission risk may also vary according to gestational timing (*17,18*). For rubella, risk for adverse fetal effects is highest during the first trimester; for cytomegalovirus, risk is highest during the first trimester but is also present after exposure during the second or third trimesters (*17*,*19*). For countries currently experiencing Zika outbreaks, it will be several months before the first pregnancies during which exposure could have occurred will reach term, particularly if the critical period of pregnancy is in the first or second trimester, as our data suggest.

Our hypothetical data (Figure 2) demonstrate the time between high levels of Zika virus transmission during pregnancy and pregnancy outcomes for each weekly cohort of pregnant women. With some shifting of dates, these projections could apply to many countries in South and Central America that are currently experiencing outbreaks of Zika virus disease.

We found ecologic evidence of a temporal relationship between maternal Zika virus infection during pregnancy and congenital microcephaly in Bahia State and the possible gestational time when risk is highest (Figure 1). This relationship does not necessarily imply causality, but it does give additional credence to the pathological findings and case reports that suggest a link between Zika virus infection and microcephaly (*1*,*5*). Assessing this relationship in other states in Brazil or other locations would have been informative, but very limited data on the spread of Zika virus are available. One limitation of the projections was that the estimated epidemic curve for Bahia State was based on Salvador, the capital city, which contains only ≈18% of the population of Bahia State. It is unknown whether the timing of the outbreak in Salvador was similar to that in the remainder of the state, which served as the basis for the microcephaly case numbers. Also, the epidemic curve for Zika virus disease is not based solely on laboratory-confirmed cases, but rather it includes both suspected and confirmed cases determined primarily on the basis of clinical presentation. The microcephaly data probably include some reporting delays, especially for January and February. Moreover, these projections assume a true association between maternal Zika virus infection and infant microcephaly; other maternal cofactors, such as other infections or environmental exposures, might account for some or all of the observed temporal relationship. The effects of the imprecision of some of the factors just described are unknown. Countries that can repeat this exercise with more precise prospective data will be better able to describe the expected critical exposure window, and if risk estimates for outcomes such as microcephaly and Guillain-Barré syndrome after Zika virus infection become available, the expected number of individuals who will be affected during a certain period can be predicted.

Some of the reported cases of microcephaly included in the graph are still being assessed, and some might not meet the final case definition for microcephaly in Brazil (i.e., head circumference ≤32 cm) (*20*); increased attention to the possible association between Zika virus infection and microcephaly may have led to overascertainment. However, the rate of false-positive reports was lower in Bahia than in other states in Brazil (*21*). Data on births of infants with microcephaly were available for September 2015–February 2016, and although the data from January and February 2016 are probably not complete, they do show a decline in the number of infants born with microcephaly. Maternal–fetal transmission might result in other adverse pregnancy outcomes, and the full range of these outcomes is of interest; however, our study accounts for microcephaly only. Also, our assumption of 40-week pregnancies does not account for possible differences in gestational age or for fetal losses and miscarriages, although early case reports do not indicate high rates of prematurity (*5*). If infants with microcephaly were consistently born premature, the relevant exposure period would be delayed to include more of the second trimester.

We assumed that the birth rates in these models remain constant throughout the year, which is not true for all locations. The data for Zika virus infection and infants with microcephaly are based on dates of report, which are probably later than actual occurrence.

Despite these limitations, our assessments provide some indication that the period of highest risk might be during the first trimester or early in the second trimester of pregnancy. This assessment can help inform public health officials about risks for microcephaly and help them plan for deliveries in areas where Zika virus disease outbreaks occur. Conducting surveillance for microcephaly but also other pregnancy outcomes such as pregnancy loss and other birth defects will enable continued evaluation of any effects of Zika virus disease might have on pregnancy. These data also emphasize the role of arboviral disease–tracking activities for informing public health planning. The US Centers for Disease Control and Prevention has prepared interim guidelines for US healthcare providers who care for women who are pregnant during a Zika outbreak (*22*) as well as interim guidelines for the evaluation and testing of

infants whose mothers might have been infected with Zika virus during pregnancy (*23*).

The consequences of Zika virus infection during pregnancy are not fully understood. Given the growing evidence of an association with microcephaly (*5*,*7*,*8*), and accounting for the time lapse between disease outbreaks and the birth of any affected infants as highlighted here, it can be expected that the number of infants born with microcephaly and other adverse pregnancy outcomes will continue to rise.

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Genetic Characterization of Archived Bunyaviruses and their Potential for Emergence in Australia

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To better understand the diversity of bunyaviruses and their circulation in Australia, we sequenced 5 viruses (Gan Gan, Trubanaman, Kowanyama, Yacaaba, and Taggert) isolated and serologically identified 4 decades ago as members of the family *Bunyaviridae*. Gan Gan and Trubanaman viruses almost perfectly matched 2 recently isolated, purportedly novel viruses, Salt Ash and Murrumbidgee viruses, respectively. Kowanyama and Yacaaba viruses were identified as being related to members of a large clade containing pathogenic viruses. Taggert virus was confirmed as being a nairovirus; several viruses of this genus are pathogenic to humans. The genetic relationships and historical experimental infections in mice reveal the potential for these viruses to lead to disease emergence.

The family *Bunyaviridae* contains a diverse group of viruses; 100 species have been approved, and many others have yet to be classified (*1*). The family comprises 5 genera: *Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, *Hantavirus*, and *Tospovirus* (*2*). Viruses of the first 4 genera are arthropod borne and cause infections of medical and veterinary importance; those of the last genus infect plants. Globally, these viruses are the etiologic agents of potentially fatal human infections such as Crimean-Congo hemorrhagic fever, Rift Valley fever, hantavirus pulmonary syndrome, severe fever with thrombocytopenia syndrome, and various sporadic viral encephalitides in the Americas (California encephalitis serogroup). Vectors of vertebrate-infecting bunyaviruses include mosquitoes, midges, sandflies, and ticks; rodents are involved in hantavirus transmission.

Human infections with bunyavirus are globally distributed. In Australia, serosurveys indicate that Gan Gan (GGV), Trubanaman (TRUV), and Kowanyama (KOWV) viruses can infect humans (*3*–*6*). Limited evidence indicates that bunyaviruses cause mild disease such as arthritis but that they do not present a serious threat to human health (*3*), thereby suggesting that species of bunyaviruses in Australia may be less virulent than those found elsewhere, despite their close relatedness to highly pathogenic species. An alternative hypothesis is that these viruses are poorly described and that the lack of knowledge and available diagnostic reagents are contributing to cases not being identified. Previous pathogenesis studies in murine models support this hypothesis. The symptoms of weanling mice infected with GGV, TRUV, and Yacaaba (YACV) viruses (all isolated during 1960–1980) indicate that these viruses are potentially neurovirulent (*7*). The recent discovery of a novel phlebovirus, genetically related to viruses from the Americas, that causes disease in humans illustrates the potential for emergence of viruses in that genus (*8*). Certainly, Australia harbors many other bunyaviruses for which genetic and serologic information is largely unknown (*7*). Hence, there is a need to address the dearth of knowledge of these viruses, to better understand their genetic relationships, and to facilitate the development of diagnostic reagents.

Two apparently novel mosquito-transmitted bunyaviruses, Salt Ash virus (SASHV) and Murrumbidgee virus (MURBV), were recently detected in mosquitoes collected in the Australian state of New South Wales (*9*). It was suggested that these viruses belong to the genus *Orthobunyavirus*, the first members of this genus to be sequenced outside the midge-transmitted Simbu serogroup that infect animals in Australia. In addition, a short sequence from a virus (designated Finch Creek virus) isolated from ticks collected from royal penguins on Macquarie Island, an Australian Antarctic Territory, indicated that the virus was probably a member of the genus *Nairovirus* (*10*).

Field studies are still in progress, but more work is needed to provide a better understanding of the diversity

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of bunyaviruses and their circulation. Unfortunately, obtaining funding for field studies can be difficult. A cost-effective alternative way to obtain material is to characterize archived material. Hence, for this study, we used archived isolates from field collections in combination with high-throughput sequencing technologies to expand our knowledge of bunyaviruses. This study revealed a genetic relationship between bunyaviruses from Australia and pathogenic bunyaviruses found elsewhere in the world, indicating emergence potential. More recent field isolates indicate that the described viruses are currently circulating, demonstrating the value of exploring archival collections.

Methods

Virus Collection, Isolation, and Culture

Most viruses in this study had been collected from mosquitoes and ticks of various species during 1953–1975 (Figure 1). Viruses were KOWV strain MRM1243, Taggert virus (TAGV) strain MI14850, TRUV thought to be strain MRM3630 (*11*,*12*), GGV strain NB6057, and YACV strain NB6028 (*13*). Viruses were originally isolated by use of intracerebral inoculation of mice or by culture on insect and mammalian cell lines (*13*,*14*); they were stored at -80°C until use in this study. After being thawed, isolates were grown on either C6/36 or Vero cells by using Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA) with 3% fetal bovine serum growth medium at 37°C (Vero) or 25°C (C6/36) under 5% CO_2. GGV and TRUV isolates used for sequencing were from the Elizabeth Macarthur Agriculture Institute collection; all other archived material was obtained from the Queensland Institute of Medical Research collection. KOWV was reisolated from a homogenate derived from a pool of *Anopheles meraukensis* mosquitoes collected in 2001 as inoculum (*15*). The homogenate was passaged 1 time on C6/36 cells and then 1 time on Vero cells.

Field Sampling

We also collected mosquito expectorate samples on sugar-baited sample cards (FTA cards; Whatman, Maidstone, UK) by using methods previously described (*16*). Mosquitoes were from Badu Island in the Torres Strait and Seisia and Bamaga on the Cape York Peninsula (Figure 1). We extracted samples by using a QIAamp Viral RNA Extraction Kit (QIAGEN, Valencia, CA, USA) and screened them for viral RNA by using TaqMan reverse transcription PCR (RT-PCR) primers SAVSTF 5′-CAGTTTC-TATCCTCTGGCTATTGGA-3′, SAVSTR 5′-GACG-CAATGCCTTTTTTAGATATTG-3′, and probe SAVSTP 5′-FAM-ATTCAGAGCCAAACAAGACCCTGAG-CAAG-TAMRA-3′.

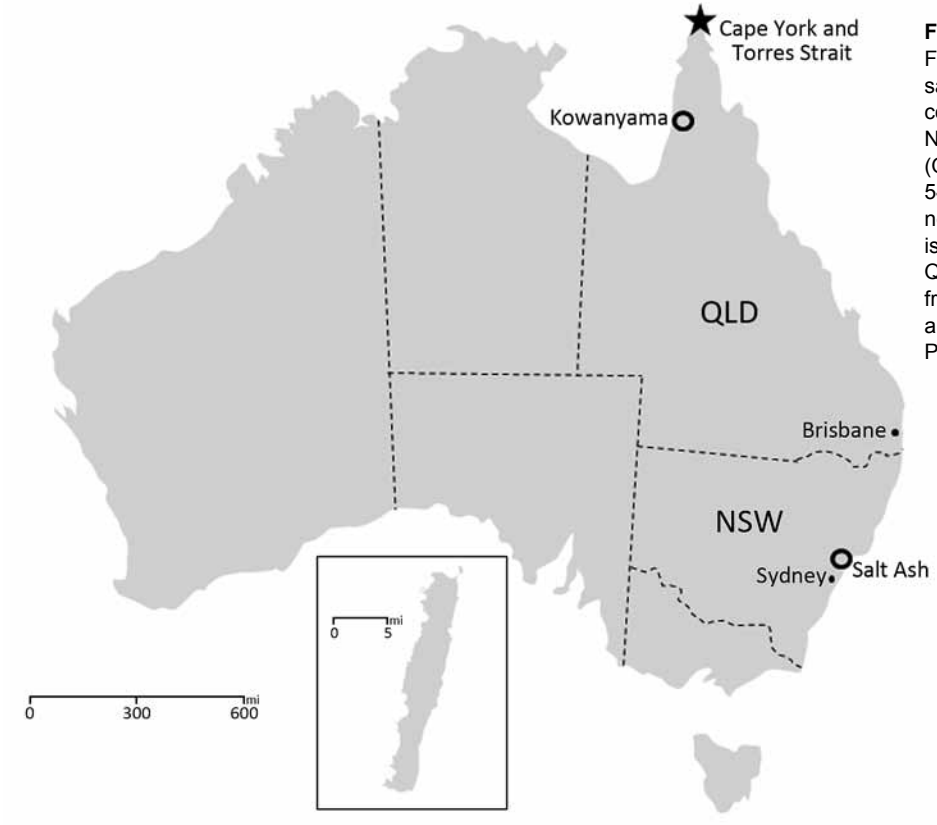

Figure 1. Bunyavirus collection and FTA card (Whatman, Maidstone, UK) sampling sites in Australia. Virus was collected from sites (open circles) in New South Wales (NSW), Queensland (QLD), and Macquarie Island (inset; 54°30S, 158°57E). Salt Ash is a town near Nelson Bay, NSW. Kowanyama is the site of the Mitchell River Mission, Queensland. FTA card sampling sites from Badu Island in the Torres Strait and Seisia and Bamaga on Cape York Peninsula are shown (star).

Archived Virus Testing

Virus was detected in samples by extraction of nucleic acids, followed by RT-PCR amplification with use of the following virus-specific primers: SAVSF1 5′-CATTGAAGTAAACCTACCAAGTGT-3′ and SAVSR1 5′-TCGAATATTGTATTATAATGATGT-3′, SAVMF1 5′-TTGTACAGTTGCTGGAAATTCAGT-3′ and SAVMR1 5′-TCTGGGTATGTTATACATATTTCT-3′, SAVLF1 5′-AGAAATAATCTAAAAAGAAGCTTA-3′and SAVLR1 5′-AAGTATAGGGTCCAATGCTGTCAA-3′ for SASHV small (S) (653 bp), medium (M) (765 bp), and large (L) (744 bp) segment amplification; and MVSF1 5′-CAGTGAGTTGAACCTAGGTAGCCT-3′ and MVSR1 5′-TCTTTTCTCTCTCCTCCTAATTTGAT-3′, MVMF1 5′-ATGCACACCTGCTTTAACTCAAAA-3′ and MVMR1 5′-GTAGGTGTGTTTATGCATATTTCA-3′, MVLF1 5′-AGGAACAATTTTAAACGTTCAATA-3′ and MVLR1 5′-TAAGATAGGATCACATGCAAATAA-3′ for MURBV S (652 bp), M (762 bp), and L (744 bp) segment amplification. We always included a no-template control.

Viral Genome Sequencing

We sequenced the viral RNA genomes as described previously (*17*). In brief, we purified virus from tissue culture supernatant by using a combination of preferential nuclease digestion and ultracentrifugation, followed by sequence-independent amplification. A library was constructed from the products and sequenced on a Personal Genome Machine (Life Technologies, Carlsbad, CA, USA) by using 316 and 318 chips.

Electron Microscopy

We clarified the tissue culture medium containing cultured virus (at 3,000 × *g* for 10 min), layered the supernatant on a 20% sucrose cushion, and subjected it to centrifugation (100,000 × *g* for 16 h at 4°C). The supernatant was discarded, and the pellet was resuspended in sterile phosphate-buffered saline (20 mL). Virus-enriched resuspension was prepared for transmission electron microscopy on glow-discharged formvar-coated copper grids and negatively stained with 1% uranyl acetate. All images were obtained on a Tecnai F30 FEG-transmission electron microscope (FEI, Hillsboro, OR, USA) operating at 300 kV.

Phylogenetic Analysis

We aligned the complete deduced amino acid sequences of each genome segment of KOWV, YACV, and TAGV with those of representative members of the *Orthobunyavirus* and *Nairovirus* genera available on GenBank. An additional nairovirus alignment was also created by using a short sequence fragment (<450 nt) of a highly conserved region of the L segment, which is the only region that has been sequenced for many nairoviruses. We generated alignments by using ClustalW implemented in Geneious version 8.1.6 (*18*), refined them manually, and removed ambiguously aligned regions by using the Gblocks program with default parameters (*19*). We constructed maximum-likelihood phylogenetic trees by using PhyML 3.0 (*20*), using the best-fit models of amino acid substitution as determined by the model selection procedures implemented through http://www.datamonkey.org (*21*). Phylogenetic relationships were determined by using a combination of nearest neighbor interchange and subtree pruning and regrafting branch swapping; 5 random starting trees were generated for each case. The phylogenetic robustness of each node was determined by using 1,000 bootstrap replicates and nearest neighbor interchange branch swapping.

Results

GGV and TRUV

Sequencing of the 5 virus isolates, previously determined by serologic testing to be members of the family *Bunyaviridae*, subsequently confirmed them to be bunyaviruses. The elucidated sequences of GGV and TRUV matched the sequences of 2 apparently novel mosquito-transmitted orthobunyaviruses identified in 2014 (*9*). These 2 viruses were GGV, which matched with SASHV, and TRUV, which matched with MURBV. Sequence alignment revealed 99% nt identity for fragments (all were >460 nt) of the S and M segments of GGV and SASHV and the S, M, and L segments of TRUV and MURBV (data not shown). This result suggests that these were in fact 2 viruses, not 4 viruses, and that the disparity resulted from different characterization methods used at the time of the separate isolations (serologic and sequence-based, respectively), which failed to identify an association. This finding is consistent with the fact that GGV was named after Gan Gan Army Base, which was located near the town of Salt Ash in New South Wales, where the virus was originally isolated from *Aedes vigilax* mosquitoes in 1970. TRUV was originally isolated from *Anopheles annulipes* mosquitoes at Mitchell River Mission in northern Queensland in 1965; Trubanaman was the original name of the Mission (*22*).

To verify that these 2 viruses had been renamed and were not simply mislabeled or incorrectly handled, we used the SASHV and MURBV GenBank genome sequences to design primer sets specific to each of the 2 viruses for detection by RT-PCR. Using assays to detect SASHV, we generated specific amplification products from material that had been designated as GGV in a separate archival collection held at the Queensland Institute of Medical Research (Figure 2) but not in material designated TRUV. Similarly, by using the MURBV assay, we generated products specific to that virus in samples designated TRUV from the Queensland Institute of Medical Research archival

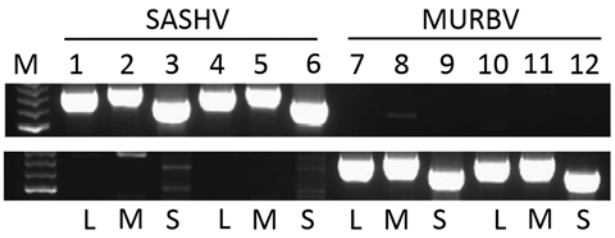

Figure 2. Sequences of Salt Ash (SASHV) and Murrumbidgee virus (MURBV) in archived stocks of Gan Gan (GGV) and Trubanaman viruses, respectively, from Australia. Archived material that was designated GGV (upper panel) and Trubanaman (lower panel) virus was extracted. This material was used in an assay designed to detect the small (S), medium (M), and large (L) segments of SASHV and MURBV viruses as indicated below the panels. Lane M, 100-bp ladder (Promega Corporation, Madison, WI, USA); lanes 1–3, GGV sample 1; lanes 4–6, GGV sample 2; lanes 7–9, MURBV sample 1; lanes 10–12, MURBV sample 2.

collection but not in material designated GGV. Hence, this evidence strongly suggests that these 2 viruses have been named twice, after independent isolations decades apart. It also indicates that both viruses have been circulating on the mainland of Australia for >40 years with little change. As part of a statewide surveillance program by the Public Health Virology Laboratory (Queensland Health Forensic and Scientific Services, Brisbane, Queensland, Australia), real-time RT-PCR detected GGV in mosquito expectorate that had been deposited onto nucleic acid sample cards (FTA cards) in a trap in the township of Seisia in northern Queensland. Hence, GGV is currently circulating in mosquito populations in that region. Unfortunately, we were not able to detect TRUV in this surveillance. However, the report by Coffey et al. (*9*) indicates that both viruses were recently circulating in New South Wales.

KOWV and YACV

Two other viruses, KOWV and YACV, were also identified as being probable members of the genus *Orthobunyavirus*. These viruses were isolated from mosquitoes: *An. annulipes* (KOWV) in 1963 and *Ae. vigilax* (YACV) in 1970. Electron microscopy revealed multiple particles for both viruses, consistent with bunyavirus morphology (Figure 3). These particles were smaller than the 80–100 nm generally observed for bunyavirus particles; the small size was attributed to overnight sedimentation through a hyperosmotic sucrose cushion.

Phylogenetic analysis revealed that these 2 viruses form a clade that is most closely related to the Gamboa group of viruses (Figure 4, panel A; online Technical Appendix Figures 1, 2, http://wwwnc.cdc.gov/EID/article/22/5/15-1566-Techapp1.pdf). KOWV and YACV are also part of a larger clade, which includes the Nyando, Wyeomyia, Bunyamwera, Bwamba, and California encephalitis groups. Members of these groups have been associated with disease in humans and animals, which suggests the potential for disease emergence associated with these 2 viruses. In combination with historical neurovirulence studies in mice (*7*), this finding reveals the pathogenic potential of these recently characterized viruses. KOWV has been recently reisolated from a pool of mosquitoes (*An. meraukensis*) from 2001 (*23*), which indicates that KOWV was circulating in Queensland in that year (data not shown).

TAGV

Phylogenetic analysis confirmed that TAGV is a nairovirus (Figure 4, panel B; online Technical Appendix Figure 3). This virus was isolated from *Ixodes uriae* ticks collected on Macquarie Island in 1972. Nairovirus particles were observed by transmission electron microscopy (Figure 3). The assembled S segment RNA sequence also matched another more recent isolate from *I. uriae* ticks from Macquarie Island, designated Finch Creek virus (*10*). The authors of that work suggested that the virus may be related to TAGV but were unable to obtain a sample to confirm the relationship. A genetic relationship to other pathogenic members of the nairoviruses was also identified. The phylogenetic analysis of TAGV S, M, and L segments, in conjunction with its match to Finch Creek virus, confirms that it is a member of the genus *Nairovirus* and that the 2 viruses are closely related, if not strains of the same virus collected decades

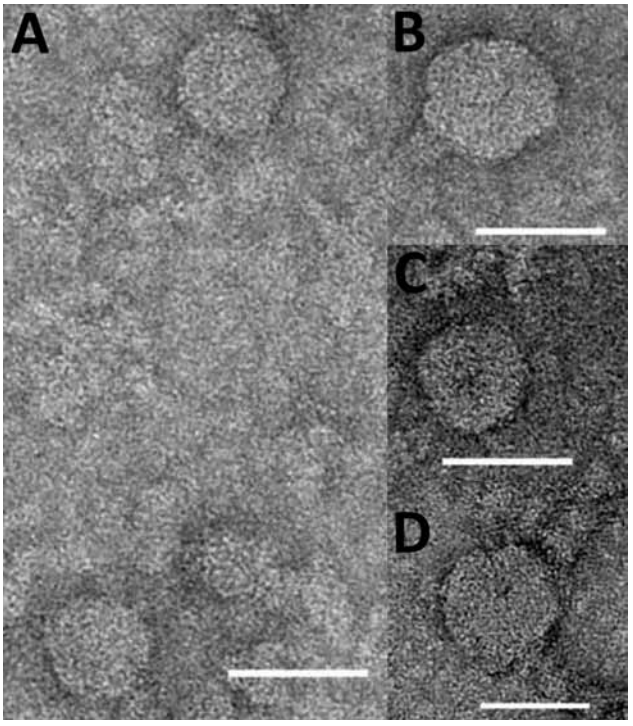

Figure 3. Negatively stained electron microscopic images of Kowanyama (A, B), Yacaaba (C), and Taggert virus (D) particles from Australia. Scale bars indicate 50 nm.

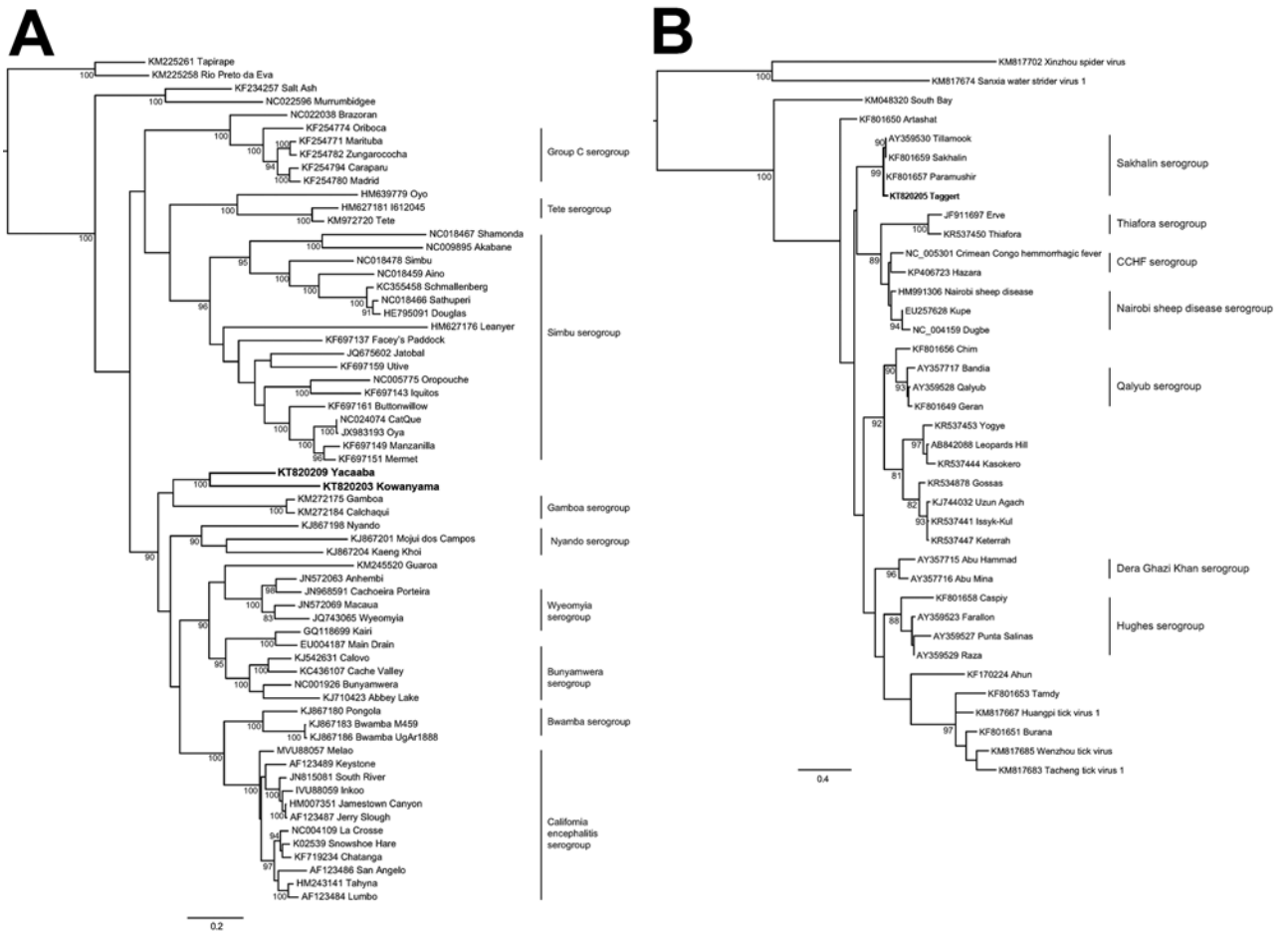

Figure 4. Phylogenetic trees including bunyaviruses from Australia. A) Relationship of Kowanyama and Yacaaba viruses (both in boldface) to other orthobunyaviruses constructed by using the predicted open reading frame sequence of the glycoprotein with a maximum-likelihood model. B) Relationship of Taggert virus (boldface) to other nairoviruses demonstrated by using the predicted open reading frame of a short fragment of the large segment (<450 nt) and a maximum-likelihood model. Virus serologic and genetic groups are shown to the right in each panel. Bootstrap values are shown as a percentage of 1,000 replicates. GenBank accession numbers are shown. CCHF, Crimean-Congo hemorrhagic fever. Scale bars indicate amino acid substitutions per site.

apart. TAGV provides another example of the problem of different characterization methods leading to duplicate designations for a single virus.

Nairoviruses have a viral homologue (vOTU) of the ovarian tumor domain superfamily of proteases, which has been linked to virus virulence (*24–26*). vOTU has broad deubiquinating activity and is able to cleave the ubiquitin-like interferon-stimulated gene protein (ISG15) involved in host immune regulation, particularly the NF-κB (nuclear factor kappa light chain enhancer of activated B cells) signaling pathway (*24–26*). An alignment of the putative RNA-dependent RNA polymerase revealed that TAGV also has this domain (Figure 5), which includes 3 highly conserved blocks. Hence, TAGV may use vOTU activity to avoid innate host immune responses; this observation adds further support to the possibility that this virus may be pathogenic.

Discussion

This study characterizes several bunyaviruses, originally collected decades ago, that are genetically similar to viruses known to be pathogenic. However, the drivers of infectious disease are not dependent solely on the genetics of the pathogen; they are also dependent on human and environmental factors, in which vector behavior plays a critical role. Previous infections of weanling mice established the neurovirulence potential of GGV, TRUV, and YACV (*7*). With regard to transmission, GGV and YACV were isolated from *Ae. vigilax* mosquitoes, which are widely distributed in coastal regions and are one of the primary vectors for Ross River virus transmission in Australia (*27*). Future transmission studies are needed to determine the vector competence of mosquito species to transmit GGV and YACV to humans, enabling an objective assessment

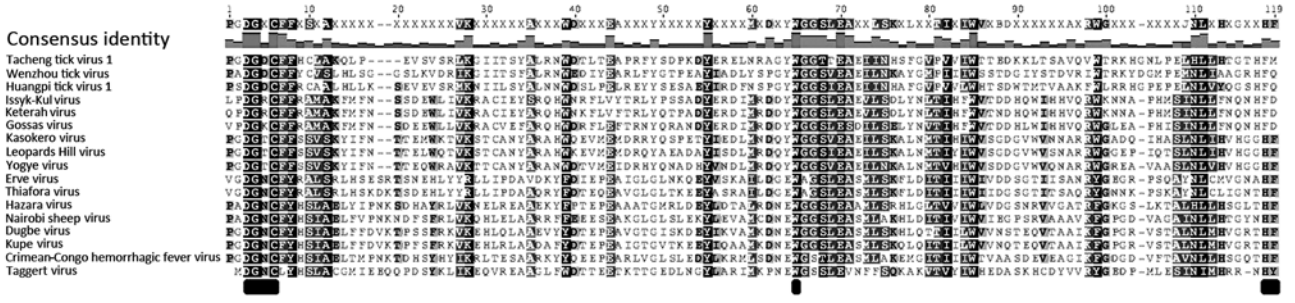

Figure 5. Taggert virus RNA-dependent RNA polymerase showing a viral homologue (vOTU) of the ovarian tumor domain. The alignment of nairoviruses shows a consensus sequence, which corresponds to the vOTU domain (*24*–*26*), which has been linked to virulence. The highly conserved residues, which include the catalytic triad residues, are indicated with a black box below each column.

of their threat to human health. The mosquito *An. annulipes*, from which TRUV and KOWV were isolated, only rarely feeds on humans (*28*) but may play a role in enzootic transmission. Furthermore, laboratory-based vector studies revealed that TRUV and KOWV replicated in *Ae. vigilax* and *Culex annulirostris* mosquitoes, respectively (*29*).

Intracerebral inoculation of TAGV into "infant" mice caused death, suggesting some level of virulence (*30*). In addition, a vOTU domain, which is a conserved region in the RNA polymerase of nairoviruses, was demonstrated to be present in TAGV. This observation and the genetic relationship between TAGV and other known pathogenic nairoviruses, suggest that TAGV may also be virulent. With regard to transmission, the *I. uriae* tick vector for TAGV has a circumpolar distribution on both hemispheres (*31*) and has been observed to occasionally bite humans (*11*). However, a limited serosurvey of Macquarie Island staff in the same study detected no antibodies to the virus in any person. The virus is yet to be detected on the Australia mainland, but it is potentially a risk to persons exposed to seabirds, the natural host of *I. uriae* ticks, which live at or have migrated through higher latitudes.

Surveillance and characterization of arboviruses are needed to better elucidate virus ecology, understand how viruses evolve, and be better prepared diagnostically if they ultimately emerge. The use of high-throughput sequencing technologies will mean that sequences for the design of diagnostic reagents to determine the virulence of these viruses can now be used. The use of archived material in this sequencing study shows the previously identified genetic stability of the arboviruses (*32*). When contemporaneous material was available, strains were found to vary by a small percentage only, for multiple genome segments, over >4 decades. This genetic stability is remarkable for RNA viruses and has previously been recognized for other arboviruses such as alphaviruses and flaviviruses. It has been attributed to the selection pressure exerted by the insect and mammalian hosts in the complete virus life cycle (*32*). It also demonstrates the value of collecting, storing, and analyzing archived material, which can reveal unexpected relationships when new technologies become available.

This work highlights an issue arising from the recent use of new high-throughput sequencing technologies, that is, the designation of recently collected virus isolates as novel material and their renaming. This issue arises from the fact that material collected before the 1980s was identified by use of serologic methods, whereas characterization of more recent isolates is primarily based on sequence analysis. To further illustrate this point, since this work was completed, another orthobunyavirus isolate from the Northern Territory in Australia, Buffalo Creek virus, was reported as being the same species as MURBV in the Mapputta group (*33*). Hence, TRUV, MURBV, and Buffalo Creek virus should all be considered the same virus. Although our study identifies a few regional examples, it is expected that this phenomenon is probably global. Workers should be aware of this possibility and should take steps to minimize the issue by sequencing archived material as well as using serologic techniques when available.

The risk for emergence of viruses, such as the ones described in this article, increases as population and growth pressures lead to development of previously undisturbed regions and concomitant exposure to native biota. This issue is especially of concern in Australia, where there is a drive to expand into the tropical northern part of the country. An additional consideration is that some persons may be exposed to insect vectors through research, mining, and other occupational activities, thereby increasing their risk for infection. In such instances, one might expect sporadic disease. The degree to which cases of sporadic disease represent a risk for spread to the community will depend on the factors affecting virulence but also on the degree of symptomatic disease and the rate at which these agents adapt to new mammalian hosts—only time will tell. But forewarned is forearmed, and surveillance remains the best preparation for future threats.

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Plasmodium falciparum In Vitro Resistance to Monodesethylamodiaquine, Dakar, Senegal, 2014

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We successfully cultured 36 *Plasmodium falciparum* isolates from blood samples of 44 malaria patients admitted to the Hôpital Principal de Dakar (Dakar, Senegal) during August–December 2014. The prevalence of isolates with in vitro reduced susceptibility was 30.6% for monodesethylamodiaquine, 52.8% for chloroquine, 44.1% for mefloquine, 16.7% for doxycycline, 11.8% for piperaquine, 8.3% for artesunate, 5.9% for pyronaridine, 2.8% for quinine and dihydroartemisinin, and 0.0% for lumefantrine. The prevalence of isolates with reduced in vitro susceptibility to the artemisinin-based combination therapy partner monodesethylamodiaquine increased from 5.6% in 2013 to 30.6% in 2014. Because of the increased prevalence of *P. falciparum* parasites with impaired in vitro susceptibility to monodesethylamodiaquine, the implementation of in vitro and in vivo surveillance of all artemisinin-based combination therapy partners is warranted.

In 2004, Senegal adopted the use of sulfadoxine/pyrimethamine with amodiaquine as the first-line therapy for malaria in response to increasing chloroquine resistance. In 2006, the National Malaria Control Program of Senegal recommended artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated malaria (*1*,*2*). The combined sulfadoxine/pyrimethamine and amodiaquine treatment was then changed to artemether/lumefantrine or artesunate/amodiaquine. The number of reports assessing levels of *Plasmodium falciparum* resistance to antimalarial drugs since the introduction of ACT in Senegal has been limited. Changes in resistance to antimalarial drugs were observed during 2008–2011 in Thiès, the third largest city in Senegal, when parasites became less susceptible to amodiaquine, artemisinin, and chloroquine (*3*). The ex vivo susceptibility to monodesethylamodiaquine, which is the active metabolite of amodiaquine, has been low and stable for the past 10 years in Dakar (6.0% in 2009, 11.8% in 2010, and 5.6% in 2013) (*4*–*6*). The prevalence of reduced susceptibility to lumefantrine remains <3.0% (*4*–*6*). To determine whether parasite susceptibility has been affected by the use of ACT, we conducted an ex vivo susceptibility study on local isolates from Dakar obtained from the Hôpital Principal de Dakar during August–December 2014. The malaria isolates were assessed for susceptibility to standard drugs, such as monodesethylamodiaquine (the active metabolite of amodiaquine), lumefantrine, chloroquine, quinine, mefloquine, artesunate, dihydroartemisinin (the active metabolite of artemisinin derivatives), doxycycline, and new antimalarial drugs (e.g., pyronaridine and piperaquine).

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Materials and Methods

Patients and Sample Collection

We obtained blood samples 44 *P. falciparum* malaria patients admitted to the Hôpital Principal de Dakar (Dakar, Senegal) during August–December 2014. Of the 44 patients, 73% were recruited from the emergency department; other patients were recruited from the intensive care unit (7%), pediatric department (13%), or other units (9%). Venous blood samples were collected from each patient by using Vacutainer acid citrate dextrose tubes (Becton Dickinson, Rutherford, NJ, USA) before treatment began. Informed verbal consent from the patients or their parents/guardians was obtained before blood collection. This study was approved by the ethics committee of the Hôpital Principal de Dakar.

For all 44 patients, no information was available on antimalarial treatment before admission. Previous intake of antimalarial drugs can affect the phenotype of parasites isolated from patients. Despite World Health Organization

recommendations, the patients were treated with quinine until November 2014, and then with artesunate or artemether/lumefantrine.

Thin blood smears were stained using a RAL kit (Réactifs RAL, Paris, France) by using eosin and methylene blue and were examined to determine *P. falciparum* density and confirm monoinfection. The level of parasitemia ranged from 0.13% to 14.13% for male patients (n = 31) and from 0.06 to 11.61% (n = 13) for female patients.

Parasitized erythrocytes were washed 3 times in RPMI 1640 medium (Invitrogen, Paisley, UK) buffered with 25 mmol/L HEPES and 25 mmol/L $NaHCO_3$. If parasitemia exceeded 0.1%, infected erythrocytes were diluted to 0.1% with uninfected erythrocytes (human blood type A+) and resuspended in RPMI 1640 medium supplemented with 10% human serum (Abcys S.A., Paris, France), for a final hematocrit of 1.5%. The susceptibility of the isolates was assessed without culture adaptation.

Drugs

Chloroquine, quinine, doxycycline, and dihydroartemisinin were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Monodesethylamodiaquine was obtained from the World Health Organization (Geneva, Switzerland). Mefloquine was purchased from Roche (Paris, France), and lumefantrine was purchased from Novartis Pharma (Basel, Switzerland). Artesunate, piperaquine, and pyronaridine were obtained from Shin Poong Pharm Co. (Seoul, Korea).

Quinine, monodesethylamodiaquine, mefloquine, dihydroartemisinin, artesunate, piperaquine, and doxycycline were dissolved in methanol and then diluted in water to final concentrations ranging from 6 nmol/L to 3,149 nmol/L for quinine, 1.9 nmol/L to 1,988 nmol/L for monodesethylamodiaquine, 1.5 nmol/L to 392 nmol/L for mefloquine, 0.1 nmol/L to 107 nmol/L for dihydroartemisinin, 0.1 nmol/L to 98 nmol/L for artesunate, 1.9 nmol/L to 998 nmol/L for piperaquine and 0.1 μmol/L to 502 μmol/L for doxycycline. Chloroquine and pyronaridine were resuspended and diluted in water to final concentrations ranging from 6 nmol/L to 3,231 nmol/L and 0.4 nmol/L to 199 nmol/L, respectively. Lumefantrine was resuspended and diluted in ethanol to obtain final concentrations ranging from 0.6 nmol/L to 310 nmol/L.

We tested and validated batches of plates on the chloroquine-resistant W2 strain (Indochina) (Malaria Research and Reference Reagent Resource Center, Manassas, VA, USA) in 5 independent experiments. The clonality of the W2 strain was verified every 15 days by using PCR genotyping of the polymorphic genetic markers *msp1* and *msp2* and microsatellite loci (*7*,*8*) and annually by an independent laboratory from the Worldwide Antimalarial Resistance Network.

Ex Vivo Assay

For the in vitro microtests, we aliquated 100 μL of parasitized red blood cells (final parasitemia 0.1%, final hematocrit 1.5%) into 96-well plates predosed with antimalarial drugs (monodesethylamodiaquine, lumefantrine, chloroquine, quinine, mefloquine, dihydroartemisinin, artesunate, piperaquine, pyronaridine, and doxycycline). The plates were incubated in a sealed bag for 72 hours at 37°C with atmospheric generators for capnophilic bacteria by using Genbag CO_2 at 5% CO_2 and 15% O_2 (BioMérieux, Marcy l'Etoile, France) (*9*).

After thawing the plates, we homogenized hemolyzed cultures by vortexing the plates. The success of the drug susceptibility assay and the appropriate volume of hemolyzed culture to use for each assay were determined for each clinical isolate during a preliminary histidine-rich protein 2 ELISA. Both the pretest and subsequent ELISA tests were performed using a commercial kit (Malaria Ag Celisa, Cellabs PTY LTD, Brookvale, Australia) in accordance with the manufacturer's recommendations. The optical density (OD) of each sample was measured with a spectrophotometer (Multiskan EX, Thermo Scientific, Vantaa, Finland).

The 50% inhibitory concentration (IC_{50}) for each of the 10 drugs was calculated with the inhibitory sigmoid E_{max} model, which estimated the IC_{50} through nonlinear regression by using a standard function of the R software (ICEstimator version 1.2, http://www.antimalarial-icestimator.net) (*10*). IC_{50} values were validated only if the OD ratio (OD at zero concentration / OD at maximum concentration) was >1.6 and the 95% CI of the IC_{50} estimation was <2.0 (*10*). The cutoff values for in vitro resistance or reduced susceptibility were as follows: 77 nmol/L for chloroquine, 61 nmol/L for monodesethylamodiaquine, 115 nmol/L for lumefantrine, 12 nmol/L for dihydroartemisinin, 12 nmol/L for artesunate, 611 nmol/L for quinine, 30 nmol/L for mefloquine, 135 nmol/L for piperaquine, 60 nmol/L for pyronaridine, and 37 μmol/L for doxycycline (*4*,*11*).

Data and Statistical Analysis

IC_{50} values were analyzed after logarithmic transformation. Values were expressed as the geometric mean of the IC_{50} with 95% CI.

Results

From the blood samples collected from the 44 *P. falciparum* malaria patients admitted to the Hôpital Principal de Dakar during August–December 2014, we successfully cultured a total of 36 isolates, and calculated the average parameter estimates for the 10 antimalarial drugs (Table). Only 44 *P. falciparum* malaria cases were reported in Dakar during the 4-month study period; a 27.6% decrease in malaria prevalence occurred in Senegal during 2013–2014 (*12*).

Table. Ex vivo susceptibility to standard antimalarial drugs of 36 *Plasmodium falciparum* isolates from 44 malaria patients compared with a *P. falciparum* W2 clone tested under the same conditions, Hôpital Principal de Dakar, Dakar, Senegal, August 2014–December 2014*

Antimalarial drug	Geometric mean IC_{50} (95% CI)†		Ratio of geometric mean IC_{50} (isolate/W2)	Cutoff for reduced susceptibility†	% Isolates with reduced susceptibility (no./no. tested)
	Isolates	W2 clone			
Monodesethylamodiaquine	25.3 (16.9–38.0)	70 (66–74)	0.36	61	30.6 (11/36)
Lumefantrine	6.8 (4.4–10.8)	15.4 (11.7–20.3)	0.44	115	0 (0/36)
Chloroquine	64.6 (46.2–90.2)	254 (234–276)	0.25	77	52.8 (19/36)
Mefloquine	22.6 (16.9–30.3)	12.7 (11.5–14.1)	1.78	30	44.1 (15/34)
Quinine	80.2 (54.4–118.2)	262 (247–278)	0.31	611	2.8 (1/36)
Piperaquine	36.4 (26.2–50.6)	34.8 (31.9–37.9)	1.05	135	11.8 (4/34)
Pyronaridine	10.5 (7.8–14.1)	26.0 (23.9–28.3)	0.40	60	5.9 (2/34)
Dihydroartemisinin	1.8 (1.17–2.77)	1.26 (1.05–1.57)	1.43	12	2.8 (1/36)
Artesunate	2.5 (1.6–3.7)	1.19 (1.03–1.41)	2.10	12	8.3 (3/36)
Doxycycline	8.5 (5.6–12.7)	10.4 (9.2–11.7)	0.82	37	16.7 (6/33)

*The geometric mean IC_{50} values for W2 are the results of 5 independent experiments, in which batches of plates were tested and validated on the chloroquine-resistant W2 strain (Indochina). IC_{50}, 50% inhibitory concentration.
†All IC_{50} values are given in nmol/L except those for doxycycline, which are given in μmol/L.

The prevalence of isolates with in vitro reduced susceptibility was 30.6% for monodesethylamodiaquine, 52.8% for chloroquine, 44.1% for mefloquine, 16.7% for doxycycline, 11.8% for piperaquine, 8.3.0% for artesunate, 5.9% for pyronaridine, 2.8% for quinine and dihydroartemisinin, and 0% for lumefantrine. The prevalence of isolates with in vitro reduced susceptibility to monodesethylamodiaquine increased significantly, from 5.6% in 2013 (*6*) to 30.6% in 2014 ($p = 0.04$ by Pearson χ^2 test). Six isolates had high monodesethylamodiaquine IC_{50}, defined as >100 nmol/L (101 nmol/L, 108 nmol/L, 140 nmol/L, 158 nmol/L, 161 nmol/L, and 227 nmol/L).

Discussion

Longitudinal in vitro analysis of the susceptibility of *P. falciparum* isolates to antimalarial drugs has 3 benefits (*13*). First, this approach enables assessment of the response of clinical isolates to individual drugs regardless of host factors that influence drug efficacy in vivo. The response of a patient to drug treatment is complex and reflects host factors and intrinsic responses of the parasite to the drug. This approach enables surveillance for resistance to both components of a drug combination such as ACT. In vitro testing is the only method that is currently available to provide clear early warning of impending resistance to the components of ACT. Second, tracking the progressive decline in drug susceptibility from the same site is likely to be the most sensitive method to identify growing resistance in a parasite population. Third, strains with reduced susceptibilities can be established in continuous culture as stable reference lines to provide the tools needed to investigate novel molecular mechanisms and to define the baseline of susceptibility to a new drug.

In vitro analysis of cross-resistance among drugs is crucial to avoid development or introduction of new drugs to which parasites are already resistant. It is also important to use the molecules that actually act in humans (i.e., the antimalarial drugs themselves) if they act directly without metabolization or with their active metabolites (e.g., dihydroartemisinin for artemisinin derivatives or monodesethylamodiaquine for amodiaquine).

In the absence of standardized ex vivo and in vitro tests, it is very difficult to compare data from different laboratories' IC_{50} and cutoff values for in vitro resistance are specific to the methodology. The in vitro effects and the IC_{50} values for antimalarial drugs depend on incubation conditions (*14*,*15*), gas conditions (e.g., the effects of O_2 and CO_2) (*9*,*16*), and methodology (e.g., use of an isotopic test vs. an immunoenzymatic test) (*17*). These differences in methodology must be taken into account when comparing and analyzing resistance data from different studies.

Our methodology was the same as that used during 2013–2014 (*6*), which enables comparison of the data. In addition, the W2 clone was used as an internal control in both studies. Comparison of W2 susceptibility data for the 10 antimalarial drugs in 2014 to those of previous years indicated no significant difference between the 2 studies in terms of response to antimalarial drugs ($0.45<p<0.91$).

The prevalence of isolates with in vitro reduced susceptibility to monodesethylamodiaquine increased significantly from 5.6% in 2013 (*6*) to 30.6% in 2014 ($p = 0.04$). In the absence of a significant difference in W2 responses to monodesethylamodiaquine between the 2 studies, the increase in the IC_{50} geometric mean and the prevalence of reduced in vitro susceptibility are attributable to the evolution of monodesethylamodiaquine susceptibility and not differences in methodology. There are 2 hypotheses that might explain the observed increase: 1) the use of artesunate/amodiaquine in Senegal generated the emergence of amodiaquine-resistant parasites (*1*,*2*), or 2) cross-resistance has occurred between chloroquine and monodesethylamodiaquine (*4*,*18*). A decrease in chloroquine resistance that was observed in Dakar during 2009–2011 parallels the withdrawal of chloroquine

treatment (*4*,*5*). However, the prevalence of in vitro resistance to chloroquine increased again in Dakar to 50% in 2013 (*6*) and 52.8% in 2014. This phenomenon had already been observed in the Dakar suburb of Pikine, where malaria parasites demonstrated an increase in the *pfcrt* 76T mutation involved in chloroquine resistance (*19*). During 2011–2012, the efficacy of artesunate/amodiaquine was 99.3% in Senegal (*20*).

The other ACT first-line treatment for uncomplicated *P. falciparum* malaria in Senegal is the combination of artemether and lumefantrine. No isolates with reduced susceptibility to lumefantrine have been detected, and prevalence of isolates with reduced susceptibility to lumefantrine has remained <3% in Dakar since the introduction of ACT (*4*–*6*). During 2011–2012, the efficacy of artemether/lumefantrine was 100% in Senegal (*20*). At the Hôpital Principal de Dakar, the patients from this study were treated with quinine until November 2014. The patients were then treated with artesunate or artemether/lumefantrine. All 44 of the patients fully recovered.

A new ACT second-line treatment for uncomplicated *P. falciparum* malaria is the combination of dihydroartemisinin and piperaquine. During 2011–2012, the efficacy of dihydroartemisinin/piperaquine was 100% in Senegal (*20*). The geometric mean IC_{50} values for piperaquine (34.8 nmol/L) observed in Dakar in 2014 were comparable to those observed in 2013 (32.2 nmol/L) (*6*). The prevalence of isolates with reduced susceptibility to piperaquine was 11.8% in 2014 in Dakar.

The pyronaridine/artesunate combination is one of the most recent ACT combinations to be considered and is currently under development. The prevalence of isolates with reduced susceptibility to pyronaridine was 5.9% in 2014 in Dakar. The geometric mean IC_{50} values for pyronaridine (10.5 nmol/L) observed in Dakar in 2014 were higher than those observed in Dakar in 2013 (5.8 nmol/L) (*6*) or in Dielmo in 1996 and 1997 (3.8 nmol/L and 4.52 nmol/L) (*21*,*22*).

The present study showed that 2.8% and 8.3% of the isolates in 2014 were less susceptible to dihydroartemisinin and artesunate, respectively. Previous studies found that no parasites were resistant to these 2 drugs in Dakar (*4*–*6*,*23*). However, the standard in vitro test was not adapted to follow resistance to artemisinin derivatives. The clinical resistance to artemisinin was manifested by an increase in the ring-stage survival rate after contact with artemisinin (*24*).

The prevalence of isolates with reduced susceptibility to mefloquine remained high in Dakar in 2014 (44.1%) compared with prevalences observed in 2001 (17%) and 2002 (13%) (*23*,*25*) but was relatively stable compared with 2009 (50%–62%) (*4*–*6*). Clinical trials are in progress to evaluate the efficacy of mefloquine for intermittent preventive treatment of infants and pregnant women with *P. falciparum* malaria. Nevertheless, mefloquine has been used relatively infrequently in Africa compared with Asia.

In 2014, 2.8% of the isolates showed low reduced susceptibility to quinine. This finding is consistent with those of previous studies conducted in Dakar (*4*–*6*,*23*,*25*). All of the patients treated with quinine until November 2014 fully recovered.

The prevalence of parasites with reduced susceptibility to doxycycline increased slightly in 2014 (16.7%) compared with previous years (10.3%–12.0%). However, the geometric mean IC_{50} was lower (8.5 μmol/L for 2014 vs. 9.2 μmol/L for 2010 and 11.6 μmol/L for 2009) (*4*,*5*).

Because of the short half-life of artemisinin derivatives, they have been paired with longer-lived partners, such as amodiaquine, lumefantrine, or piperaquine, for longer drug action and prophylaxis against reinfecting parasites. Because of the increased prevalence of *P. falciparum* parasites with impaired in vitro susceptibility to monodesethylamodiaquine in Dakar in 2014, implementation of in vitro and in vivo surveillance of all ACT partners is warranted. This in vitro reduced susceptibility to monodesethylamodiaquine might soon affect the in vivo efficacy of artesunate/amodiaquine, which will become the equivalent of a monotherapy using only artesunate if this in vitro resistance is confirmed in the future, especially because resistance to artesunate has already emerged in Asia (e.g., Cambodia, Thailand, Myanmar, Vietnam, China, and India) (*26*). In addition, in Senegal, it will be a priority to identify mutations in the *PF3D7_1343700* kelch propeller domain. In southern Asia, these mutations have been associated with in vitro resistance to artemisinin and delayed clearance after artemisinin treatment (*27*).

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Astrovirus MLB2, a New Gastroenteric Virus Associated with Meningitis and Disseminated Infection

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Next-generation sequencing has identified novel astroviruses for which a pathogenic role is not clearly defined. We identified astrovirus MLB2 infection in an immunocompetent case-patient and an immunocompromised patient who experienced diverse clinical manifestations, notably, meningitis and disseminated infection. The initial case-patient was identified by next-generation sequencing, which revealed astrovirus MLB2 RNA in cerebrospinal fluid, plasma, urine, and anal swab specimens. We then used specific real-time reverse transcription PCR to screen 943 fecal and 424 cerebrospinal fluid samples from hospitalized patients and identified a second case of meningitis, with positive results for the agent in the patient's feces and plasma. This screening revealed 5 additional positive fecal samples: 1 from an infant with acute diarrhea and 4 from children who had received transplants. Our findings demonstrate that astrovirus MLB2, which is highly prevalent in feces, can disseminate outside the digestive tract and is an unrecognized cause of central nervous system infection.

Astroviruses, family *Astroviridae*, are small, nonenveloped, single-stranded RNA viruses. The family comprises 2 genera: *Mamastrovirus* species infect mammals, including humans, and *Avastrovirus* species infect poultry and other birds. Human astroviruses (HAstVs) were first identified in 1975 (*1*); until recently, only classic HAstVs that belonged to the species *Mamastrovirus* (MAstV) *1* were recognized as human pathogens. HAstVs contribute to ≈10% of nonbacterial, sporadic gastroenteritis in children, with the highest prevalence observed in community healthcare centers (*2*,*3*). Symptoms are generally mild, with patient hospitalization usually not required; asymptomatic carriage has been described in 2% of children (*4*).

Screening of fecal samples from persons with diarrhea and control samples in different parts of the world by unbiased next-generation sequencing (NGS) or reverse transcription PCR (RT-PCR) has revealed the sporadic presence of members of the *Astroviridae* family, previously unrecognized in humans, that are phylogenetically substantially distant from classic HAstVs (*3*,*5*–*9*). These viruses have been named HAstV-VA/HMO and HAstV-MLB, for Virginia, human-mink-ovine, and Melbourne, respectively, according to the place where they were first identified and their close phylogenetic distance to animal astroviruses; these viruses belong to distinct species (*10*).

Cellular receptors and targeted cells for these viruses are unknown and, to date, novel astroviruses have not been culturable. Although the primary site of astroviral replication seems to be the gastrointestinal tract, disseminated diseases and encephalitis have been associated with infection with classic and nonclassic astroviruses (*11*–*16*). In animals, astroviruses also have the potential to target other organs; hepatitis and nephritis have been observed in avian infections (*4*,*17*).

These observations point to the noteworthy genetic diversity of astroviruses and their probable cross-species transmission. Nonetheless, clinical disease associated with new astrovirus variants remains to be confirmed (*9*,*18*). Although HAstV-MLB has been recovered from fecal samples of patients with acute flaccid paralysis (*6*), to our knowledge, no reports have documented this variant in cerebrospinal fluid (CSF) or central nervous system (CNS) tissue samples.

In June 2013, we launched a single-center prospective study using NGS to determine potential viral etiologic agents of meningoencephalitic and respiratory syndromes.

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Yet, in ≈50% of meningoencephalitis cases clinically suspected to be of viral origin, origins remain undetermined, despite comprehensive microbiologic investigations (*19*,*20*). We report the detection, in the context of this project, of an astrovirus MLB2 in the CSF of an immunocompetent adult patient with acute meningitis who was hospitalized at the University of Geneva Hospitals, Geneva, Switzerland, and the results of the pilot prevalence study and clinical investigation that this discovery triggered.

Materials and Methods

Virus Discovery Study

The virus discovery study and the pilot retrospective prevalence study it generated were approved by the University of Geneva Hospitals (CCER no. 13-075), and informed consent was obtained from the case-patient. This single-center epidemiologic study is ongoing (online Technical Appendix 1, http://wwwnc.cdc.gov/EID/article/22/5/15-1807-Techapp1.pdf).

High-Throughput Sequencing and Sequence Analysis

High-throughput sequencing (RNA-seq library preparation, paired-end sequencing by using the 100-bp protocol with indexing on a HiSeq 2500 [Illumina, San Diego, CA, USA]) was performed directly on the case-patient's CSF, plasma, urine, and anal swab specimen, and we analyzed results using the ezVIR pipeline as described (*21*). Of note, a DNA-seq library was also prepared and analyzed for the screening of CSF specimens of the virus discovery study.

We used high-throughput sequencing data from the anal swab specimen to obtain a MLB2 consensus sequence by aligning the reads from the ezVIR output with those of the MLB2 genome Bowtie2 (*22*) and then assembling them using Sparse Assembler (*23*). We used the full sequence (GenBank accession no. KT224358) and the capsid region (corresponding to nt 3831–6069 on the consensus sequence and nt 3843–6080 on the reference sequence) to perform a phylogenetic analysis. We made multiple alignments using multiple alignment with the fast Fourier transform (*24*) and built the tree using IQTree (*25*), with 10,000 bootstrap replicates. The tree was created with Evolview (*26*) using reference strains from GenBank (online Technical Appendix 2 Tables 1, 2, http://wwwnc.cdc.gov/EID/article/22/5/15-1807-Techapp2.pdf).

Extraction and Construction of Specific Real-Time RT-PCR

We spiked 190-μL patient specimens of CSF, plasma, urine, anal swab, and nasopharyngeal aspirates with 10 μL of standardized canine distemper virus of known concentration (*27*) and extracted RNA with the NucliSENS easyMAG (bioMérieux, Geneva, Switzerland) nucleic acid kit in an elution volume of 25 μL, according to the manufacturer's instructions. We directly used extracted RNA for astrovirus MLB2–specific real-time RT-PCR screening analysis using an assay described by Holtz et al. (*11*). We performed PCR assay reaction using the QuantiTect Probe RT-PCR Kit (QIAGEN, Valencia, CA, USA) on a StepOnePlus instrument (Applied Biosystems, Rotkreuz, Switzerland) under the following cycling conditions: 50°C for 30 min, 95°C for 15 min, 45 cycles of 15 s at 94°C, and 1 min at 55°C. Data were analyzed with the StepOne software V.2 (Applied Biosystems). Analytical sensitivity was assessed with a plasmid including the target region and showed a limit of detection corresponding to 25 copies/reaction. We further analyzed positive specimens for confirmation with a second real-time RT-PCR targeting the viral RNA-dependent RNA polymerase region (forward primer 5′-TCCCTTCTGGTGAGGTCACTCT-3′, reverse primer 5′-AGGCTTGCAACCAATAGTTAATCAT-3′, and probe 5′-FAM-AACCGTGGTAATCCATCCGGTCAAATAT-CA-TAMRA-3′) under the following cycling conditions: 50°C for 30 min, 95°C for 15 min, 45 cycles of 15 s at 94°C, and 1 min at 60°C.

Pilot Prevalence Study

To estimate the local prevalence of this novel astrovirus, we tested some CSF specimens, including all of those with a total leukocyte count of >5 cells/μL, collected from April 2013 through April 2015, and all fecal specimens collected from August 2014 through August 2015 with the astrovirus MLB2–specific real-time RT-PCR targeting the capsid gene. Specimens were collected from pediatric and adult patients hospitalized at the University of Geneva Hospitals, a 1,900-bed tertiary-care medical center, and sent to the center's laboratory of virology for any clinical purpose. All samples had been stored at –80°C.

Results

Astrovirus MLB2 in Case-Patient

The CSF collected from a patient with acute meningitis who was enrolled in the virus discovery study tested positive for astrovirus MLB2 (Figure 1) with a total of 155 specific reads (35% genome coverage; total covered, 2,183 bp). Reads did not map to other RNA viruses, and DNA sequencing revealed no reads for bacterial or viral pathogens. For this case-patient, astrovirus MLB2–specific reads were further detected by NGS in the following acute-phase specimens: anal swab (70,890 reads, 9,340 after duplicate removal; 99% genome coverage; total covered, 6,107 bp); plasma (18 reads, 5% genome coverage; total covered, 336 bp); and urine (16 reads; 1% genome coverage; total covered, 120 bp) (Figure 2, panel A).

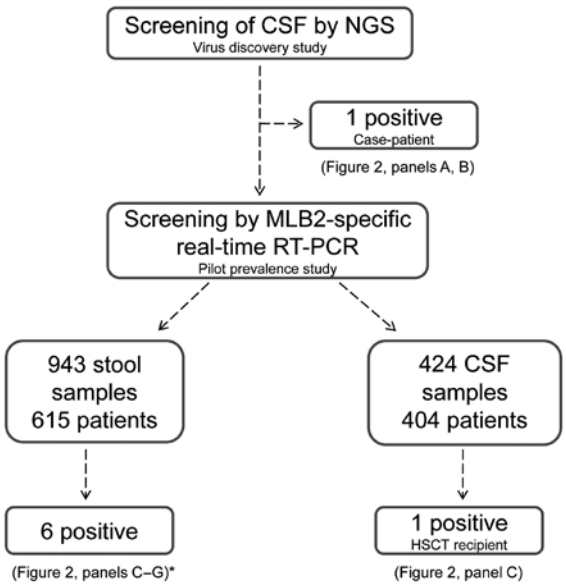

Figure 1. Flowchart of study using NGS to determine potential viral etiologic agents of meningoencephalitic and respiratory syndromes, Geneva, Switzerland, 2014. *The diarrheic immunocompetent infant is not represented in Figure 2. CSF, cerebrospinal fluid; NGS, next-generation sequencing; RT-PCR, reverse transcription PCR.

CSF obtained at hospital admission was confirmed positive by astrovirus MLB2–specific real-time RT-PCR targeting the capsid gene (*11*). Anal swab and urine specimens collected during the acute phase were also confirmed positive by astrovirus MLB2–specific RT-PCR, with the highest viral load found in the anal swab specimen. The plasma specimen drawn at admission showed a low viremia level, whereas plasma and additional CSF collected during the convalescent phase 5 and 2 days later, respectively, were negative (Figure 2, panel B). A second confirmatory assay targeting the RNA-dependent RNA polymerase gene confirmed all positive results (data not shown). Plasma and fecal specimens collected from the patient 8 months later were negative (Figure 2, panel B).

Phylogenetic analysis was performed on the full-length genome and on capsid sequences (Figure 3; online Technical Appendix 2 Figure, Table 1). Astrovirus MLB2 Geneva 2014 shows 98.5% nucleotide sequence identity homology with the complete genome of an astrovirus MLB2 isolate MLB2/human/Stl/WD0559/2008 detected in a viremic child in St. Louis, Missouri, USA, in 2011 (*11*).

Pilot Prevalence Study

We screened 943 fecal specimens from 615 unique patients; specimens from 6 patients (1%) were positive for astrovirus MLB2 by the 2 RT-PCR assays, bringing the overall number of positive patients to 7. Except for 1 immunocompetent infant who was brought for treatment with diarrhea of 15 days' duration, all patients were highly immunocompromised: 1 was an adult recipient of a hematopoietic stem cell transplant (HSCT) (Figure 2, panel C) and 4 were children who received solid organ transplants (Figure 2, panels D–G). Two patients had concomitant viremia (Figure 2, panels C and D), and 1 had 2 astrovirus MLB2–positive fecal samples, collected 2 weeks apart (Figure 2, panel G). Most patients had past or current digestive tract symptoms; the immunocompetent infant with diarrhea had no other digestive pathogen retrieved, and no other explanation was found for his symptoms. One child who had received a transplant experienced concomitant and recurrent *Clostridium difficile* infection, and adenovirus DNA was found in his feces.

Among 424 CSF specimens collected from 404 patients hospitalized in the 2 previous years, 1 supplementary specimen was positive for astrovirus MLB2. The patient was the HSCT recipient whose feces had also been screened positive (Figure 2, panel C). Of note, we detected astrovirus MLB2 RNA in CSF from this patient over a 3-month period and intermittently in plasma specimens from this patient over a 2-month period.

Meningitis: Clinical Case Descriptions

The case-patient (Figure 2, panels A, B) had been enrolled in the virus discovery study. In September 2014, this previously healthy 21-year-old woman sought treatment for an unusually severe headache and fever of a few hours' duration. She lived in a rural area, had 2 housecats, and had recently traveled to Portugal. She worked at a children's daycare center. Physical examination revealed neck stiffness without focal neurologic deficits. Blood laboratory test results were within reference limits; leukocyte count was 4.2×10^9 cells/L, and C-reactive protein level was 27 nmol/L. Analysis of CSF obtained by lumbar puncture (LP) at admission revealed clear fluid yet an abnormally high leukocyte count of 915 cells/μL (reference range 0–5 cells/mL), with 93% neutrophils; slightly elevated protein (73 mg/dL, reference range 15–45 mg/dL); and a CSF/plasma glucose ratio of 0.53. The patient was admitted and ceftriaxone and acyclovir were administered empirically. Bacterial CSF cultures remained negative, as did viral real-time RT-PCR assays targeting herpes simplex virus, varicella-zoster virus, enteroviruses, parechovirus, and Toscana virus. Serologic testing for HIV, *Treponema pallidum* (syphilis), tick-borne encephalitis virus, and *Borrelia burgdorferi* (Lyme disease) were negative, as were blood cultures. The patient underwent repeat LP 4 days after admission; the CSF leukocyte count had decreased to 47 cells/μL with a shift toward lymphocytic predominance (92%), whereas protein levels had returned to reference range (21 mg/dL). Real-time PCR results for herpes simplex virus and

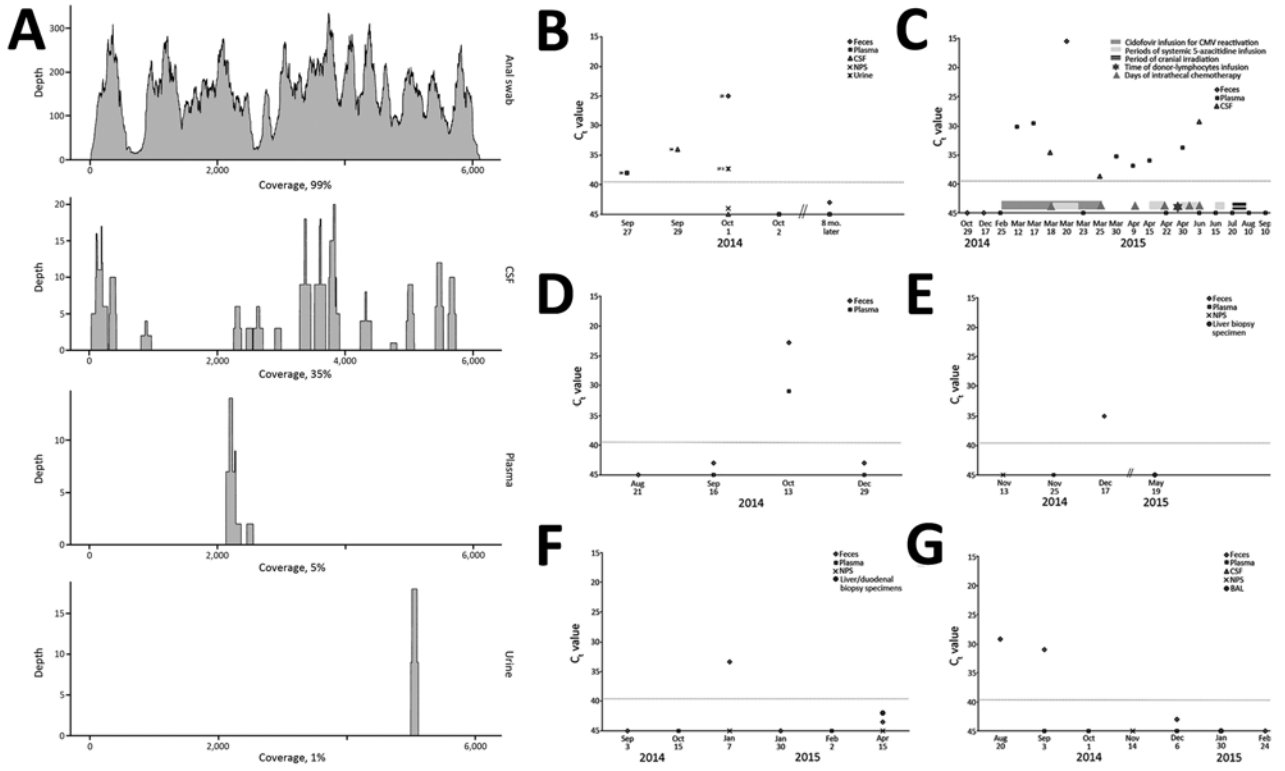

Figure 2. Details of the cases of astrovirus MLB2 infection, Geneva, Switzerland, 2014. A) Next-generation sequencing results for the case-patient. Read coverage histogram is shown for each specimen analyzed. Percentage of genome coverage is also indicated. B–C) Real-time RT-PCR analysis results for the case-patient (B) and the HSCT recipient (C); D–G) real-time RT-PCR analysis results for the solid organ transplant pediatric recipients: liver transplant (D–F) and kidney transplant (G). Dashed lines represent the limit of PCR positivity (cycle threshold 40). CSF, cerebrospinal fluid; NPS, nasopharyngeal swab; BAL, bronchoalveolar lavage.

varicella-zoster virus remained negative on the second CSF analysis, and acyclovir was discontinued. Real-time PCR results for *Streptococcus pneumoniae* and *Neisseria meningitidis* was also negative, and ceftriaxone was discontinued after 7 days. The patient continued to improve; she was discharged 10 days after admission with a presumptive diagnosis of viral meningitis.

The second patient (Figure 2, panel C) was a HSCT recipient screened by the pilot prevalence study. He was a 37-year-old man who underwent HSCT on October 2014 for acute myeloid leukemia. His household included young children. In March 2015, he experienced a headache and was ultimately given a diagnosis of a leukemic relapse, including meningeal involvement with a CSF leukocyte count of 2,240 cells/μL and a flow cytometry result confirming that 90% were blast cells. Magnetic resonance imaging showed signs of meningeal leukemic infiltration without cerebral involvement. The patient subsequently received 6 cycles of intrathecal chemotherapy and 4 cycles of 5-azacitidine, which led to remission. LPs on March 25 and June 3 revealed CSF leukocyte counts within normal limits that were nonetheless difficult to interpret given the patient's marked systemic leukopenia (0.7 and 1.1 × 10^9 cells/L, respectively). At that time, the patient experienced episodes of vertigo, limb weakness, lightheadedness, and recurrent headache, for which follow-up magnetic resonance imaging was performed. Although meningeal infiltration had diminished, it was still detectable. Thus, the patient underwent cranial irradiation beginning in July 2015. The patient then received a diagnosis of a second relapse of leukemia and died in December 2015.

Discussion

Detection of astrovirus MLB2 RNA in the CSF of the initial case-patient with acute meningitis highlights the conclusion that as-yet-unrecognized potential new human pathogens can be identified by means of molecular unbiased screening in appropriately targeted populations. The subsequent detection of the same virus's RNA in fecal specimens of 6 additional patients (of whom 5 were immunocompromised, 2 had viremia, and 1 had a positive CSF specimen) demonstrates that this virus circulates in the community and could be an unrecognized cause of

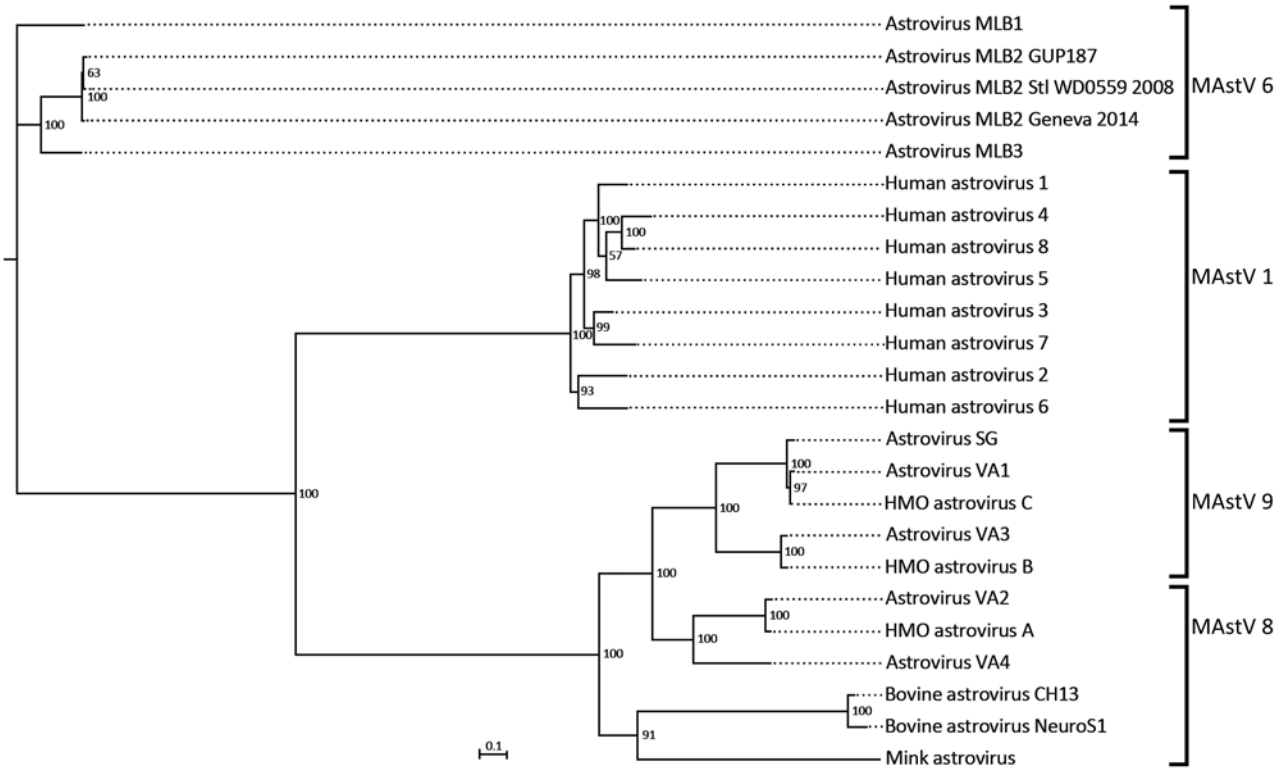

Figure 3. Phylogenetic tree constructed on the basis of full-length sequences of astroviruses and mamastroviruses. The sequence from the case-patient in this study is astrovirus MLB2 Geneva 2014. Brackets indicate the 4 Mamastrovirus species (MAstV 1, 6, 8, 9) from humans. Virus names and corresponding GenBank accession numbers are listed in online Technical Appendix 2 Table 1 (http://wwwnc.cdc.gov/EID/article/22/5/15-1807-Techapp2.pdf). Scale bar indicates nucleotide substitutions per site.

certain clinical manifestations, particularly in patients at increased risk for complications.

Although several studies have demonstrated that novel species of human astroviruses are circulating throughout the world, their associated clinical manifestations require further characterization. To the best of our knowledge, 5 cases of astrovirus CNS infection have been reported in humans, 1 caused by classic HAstV-4 and 4 caused by HAstV-VA1/HMO-C/PS (Table), but none attributed to the distant astrovirus MLB2 described here. In 2011, Wunderli et al. described a cluster of 3 children in a pediatric stem cell transplantation unit who were infected by classic HAstV-4 (*12*). Disseminated viral infection was diagnosed in 1 child who died of multiple organ failure; astrovirus was detected in several organs, including the brain and bone marrow. Similarly, HAstV-VA1/HMO-C has been detected in a few immunocompromised persons who had CNS infection and encephalitis (*13*–*16*). In animals, 2 astroviruses closely related to HAstV-VA1/HMO-C have been identified, 1 in minks with so-called shaking mink syndrome, the other in cattle with nonsuppurative encephalitis (*28*–*30*).

We have been able to partially fulfil the criteria proposed by Fredricks and Relman to show microbial disease causation on the basis of molecular tests (*31*): the high viral load observed in the fecal specimens of the 2 patients with meningitis suggests that the gastrointestinal tract is the primary site of replication, which is consistent with the tropism of this family of viruses. From the anal swab specimen of the initial case-patient, we were able to sequence the whole genome and thus demonstrate the presence of the entire virus. The transient presence of viral RNA in plasma and CSF with cycle threshold values indicating a lower viral load suggests hematogenous dissemination from the gastrointestinal tract to the meninges. With resolution of the disease, blood and fecal samples were negative for HAstV MLB2 RNA. Thus, a causal link between astrovirus MLB2 and the case-patient's acute meningitis is highly plausible. In contrast to patients in previous reports, the case-patient in our study was immunocompetent with an uncomplicated clinical course, suggesting that these viruses should probably not be considered as purely opportunistic.

In the HSCT recipient, the protracted, relatively high viral loads detected in plasma and CSF potentially mirrored

Table. Clinical cases of astrovirus infection recovered outside the digestive tract in humans by next-generation sequencing or real-time RT-PCR*

Authors (reference or figure)	Astrovirus strain	Species	Sample analyzed and results					
			Brain biopsy/CSF	Plasma/ serum	Feces	Urine	NPS	Other
Holtz et al. (*11*)	HAstV MLB2	MAstV 6	NP	+	NP	NP	+	NP
This study	HAstV MLB2	MAstV 6						
Case-patient (Figure 2, panels A, B)			+	+	+	+	–	NP
HSCT recipient (Figure 2, panel C)			+	+	+	NP	NP	NP
Patient D (Figure 2, panel D)			NP	+	+	NP	NP	NP
Wunderli et al. (*12*)	Classical HAstV serotype 4	MAstV1						
Patient 1			–/NP	+	+	–/NP	+	-/NP
Patient 2			+	+	+	–/NP	–/NP	+†
Patient 3			+	–/NP	–/NP	–/NP	+	–/NP
Quan et al. (*13*)	HAstV-PS	MAstV 9	+	NP	NP	NP	NP	–‡
Brown et al. (*14*)	HAstV-VA1/HMO-C-UK1	MAstV 9	+	+	+	NP	NP	NP
Naccache et al. (*15*)	HAstV-VA1/HMO-C-UK1	MAstV 9	+	NP	NP	NP	NP	NP
Fremond et al. (*16*)	HAstV-VA1/HMO-C-PA	MAstV 9	+	NP	NP	NP	NP	NP

*RT-PCR, reverse transcription PCR; CSF, cerebrospinal fluid; NPS, nasopharyngeal swab; HAstV, human astrovirus; MAstV, mamastrovirus; NP; not performed; +, positive; –, negative; HSCT, human stem cell transplant.
†Vesicle swab, heart, lung, spleen, bone marrow, kidney, small intestine.
‡Kidney, liver, spleen.

the cycles of immunosuppressive therapy he concomitantly received. Yet, other factors, such as his underlying illness or potential drug toxicity, could have caused and maintained his neurologic symptoms. Nonetheless, our observations indicate that astroviruses cause viremia and express CNS tropism; these findings provide a plausible explanation for the encephalitis cases recently described (*12–16*). The source of infection in these patients is unknown, although they may have been infected by contact with children. The additional detection of the virus in 5 fecal specimens from children supports this hypothesis. Alternatively, although no animal astrovirus MLB2 reservoir has yet been identified, zoonotic transmission remains another possibility (*17*,*32*).

We assessed the potential circulation of this unrecognized virus in humans by examining its prevalence in different biological specimens of interest. Our hospital-based investigation over a 1-year period found an incidence rate of astrovirus MLB2 infection of 1.1% (including the case-patient) in feces, which is higher than found in most other studies (*3*,*9*,*33*,*34*). In comparison, in our hospital, 1-year incidence rates of 3 other enteric viruses, noroviruses, rotaviruses, and enteroviruses, were 5.5%, 6.7%, and 2.7%, respectively. Whether the global prevalence of astrovirus MLB2 is underestimated or fluctuates from year to year remains to be determined. Unlike results from a previous report (*14*), CSF samples were successfully screened, with a positivity rate of 0.5% (2/405), which supports consideration of the virus in the investigations of unexplained CNS infection. Curiously, a classic symptom of human astrovirus infection is headache (*2*,*4*).

The pathophysiology and clinical manifestations of astrovirus MLB2 and other astroviruses require further definition. Of 7 patients with astrovirus MLB2 in feces, in only 1 patient did this finding have a clear clinical correlation with digestive symptoms. Thus, as with noroviruses (*35*), carriage may be prolonged after a subclinical or transient gastrointestinal illness or, as with classic astroviruses or enteroviruses, gastrointestinal replication and carriage may occur without digestive symptoms. Indeed, in a recent case-cohort study, astrovirus MLB2 was recovered in the feces of 8 patients who did not have overt digestive symptoms (*9*).

Additional virologic and epidemiologic investigations are required to assess our findings; however, seroresponses could not be evaluated because of the lack of an available antibody assay. In the absence of neural tissue sampling, in situ hybridization could not be considered. We could not isolate or demonstrate active viral replication because of the lack of a cell culture system for novel astroviruses. Furthermore, our RT-PCR assays were not quantitative, although cycle threshold values gave substantial information. These factors require more laboratory investigations, which are justified by the potential clinical effects of astroviruses that this study has highlighted. Finally, our prevalence study was retrospective and did not include healthy control patients, limiting our ability to draw solid conclusions with regard to associated disease patterns.

Although we do not provide evidence of disease causality for HAstV MLB2, according to classic Koch's postulates, our preliminary findings could place astrovirus MLB2 in the differential diagnosis not only of diarrhea but also of aseptic meningitis and protracted infection in highly

immunocompromised hosts. Potential determinants of extraintestinal dissemination, such as viral load kinetic, immune response, and host and viral genetic factors, require further characterization. Should further studies confirm our findings, patients with unexplained meningoencephalitis and those with severe immunosuppression should be considered for astrovirus MLB2 screening.

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Spectrum of Viral Pathogens in Blood of Malaria-Free Ill Travelers Returning to Canada

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Release date: April 14, 2016; Expiration date: April 14, 2017

Learning Objectives

Upon completion of this activity, participants will be able to:

- Describe the prevalence of specific common and emerging viral pathogens in malaria-negative specimens from ill travelers returning to Canada, based on a study
- Evaluate issues regarding detection of specific common and emerging viral pathogens in malaria-negative specimens from ill returning Canadian travelers
- Distinguish clinical issues regarding specific common and emerging viral pathogens detected in malaria-negative specimens from ill returning Canadian travelers.

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Malaria is the most common specific cause of fever in returning travelers, but many other vectorborne infections and viral infections are emerging and increasingly encountered by travelers. We documented common and emerging viral pathogens in malaria-negative specimens from ill travelers returning to Canada. Anonymized, malaria-negative specimens were examined for various viral pathogens by real-time PCR. Samples were positive for herpes simplex

viruses 1 or 2 (n = 21, 1.6%), cytomegalovirus (n = 4, 0.3%), Epstein-Barr virus (n = 194, 14.9%), dengue virus types 1–4 (n = 27, 2.1%), chikungunya virus (n = 5, 0.4%), and hepatitis A virus (n = 12, 0.9%). Travel-acquired viral pathogens were documented in >20% of malaria-negative specimens, of which 2.5% were infected with dengue and chikungunya viruses. Our findings support the anecdotal impression that these vectorborne pathogens are emerging among persons who travel from Canada to other countries.

Malaria is the most common specific cause of fever in travelers returning from tropical or subtropical travel; however, many vectorborne and viral infections are emerging in new geographic areas and are increasingly encountered by persons who travel. In multi- and single-center analyses, the top specific causes of fever in returning travelers include malaria (20%–30%), acute travelers' diarrhea or gastroenteritis (10%–20%), respiratory tract infections (10%–15%), dengue fever (5%–10%), enteric fever due to *Salmonella enterica* serotypes Typhi and Paratyphi (2%–7%), skin and soft tissue infections (2%–11%), rickettsioses (3%), acute urinary tract and sexually transmitted infections (2%–3%), viral hepatitis (3%), and mononucleosis-like syndrome (3%–25%) (*1*–*6*).

In North America, the burden of imported notifiable diseases, such as malaria (*7*) enteric fever (*8*), influenza (*9*), measles (*10*), and viral hepatitis (*9*), is reasonably well documented, but emerging imported viral infections, such as dengue virus (DENV) and chikungunya virus (CHIKV) infections, are not necessarily reportable to public health agencies (*11*). Thus, the anecdotal impression among clinicians that these illnesses are increasingly being imported is difficult to substantiate because national level statistics are lacking. Annual surveillance reports by CanTravNet indicate that before the establishment of CHIKV in the Americas in late 2013, cases of CHIKV infection were infrequently reported among travelers, but infections that did occur were mostly imported from the Indian Ocean islands and Southeast Asia (*12*,*13*). Mathematical modeling of infectious diseases predicts that importations of CHIKV infections to areas where the disease is not endemic are probably vastly underestimated and on the rise (*14*).

Moreover, as noted above, a high percentage of febrile returned travelers are ultimately diagnosed with a nonspecific viral syndrome, which tends to resolve uneventfully without therapy and remains unconfirmed by specific diagnostic tests. In our recent GeoSentinel network–wide analysis of illness in travelers returning from Sierra Leone, Liberia, or Guinea (*15*), we noted that fever was the most common reason for seeking healthcare, and the fever was most commonly due to malaria. However, "nonspecific viral syndrome" and "febrile illness not otherwise specified lasting <3 weeks" were the fourth and fifth most common diagnoses made for ill returned nonimmigrant travelers (*15*). Similarly, other studies have noted that up to 40% of febrile returned travelers receive no specific etiologic diagnosis (*16*–*18*). Thus, a substantial diagnostic gap exists, considering that fever in returning travelers may herald life-threatening, emerging infections of critical public health importance. To better understand the epidemiology of imported viral infections, we documented the spectrum of common and emerging viral pathogens in ill travelers returning to Canada who did not have malaria.

Methods

Specimens

The malaria biobank at the Public Health Ontario Laboratory (Toronto, ON, Canada) houses aliquots of denominalized, surplus whole blood (EDTA) specimens submitted for malaria diagnostic testing; specimens are maintained at −80°C. The biobank database contains denominalized demographic and clinical data (e.g., age, sex, and travel history) provided on test requisition forms as well as parasitologic data (e.g., parasitemia and malaria species) for positive specimens.

For this study, we used anonymized, surplus EDTA-containing specimens stored in the malaria biobank during May 2006–April 2007 and anonymized malaria-negative whole blood specimens that were accrued prospectively during February 2013–March 2014. Ethics approval was obtained from the Public Health Ontario Ethics Review Board. Extracted data were limited to age, sex, and travel history when provided on the standard test requisition form and included in the malaria biobank.

Before sending negative specimens to the malaria biobank, we performed routine malaria diagnostics in the clinical laboratory; these diagnostics were performed by microscopy examination of Giemsa-stained thick and thin blood films and by using the rapid diagnostic test with the BinaxNOW Malaria test kit (Alere, Scarborough, ME, USA). We confirmed all malaria results at study initiation using a multiplex real-time PCR assay (*19*). A malaria-negative specimen was defined as one for which Giemsa-stained expert microscopy and rapid diagnostic test results were negative and the patient did not have a documented prior episode of malaria in the past year. All malaria-negative specimens accrued during the enrollment period were subjected to a series of real-time PCR assays, as described in the following sections. We considered these specimens to have come from febrile or ill returned travelers because malaria is not endemic in Canada and, thus, testing for the disease necessarily implies illness after international tropical or subtropical travel. Fever is the most common complaint at the initial healthcare visit for persons diagnosed with malaria (*18*).

DNA and RNA Extraction from Whole Blood

We used the KingFisher Flex 96 Instrument (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions to separately perform DNA and RNA extractions from anonymized malaria-negative specimens. To extract DNA, we used the KingFisher Pure Blood Kit (Thermo Fisher Scientific), with the following modifications to volume requirements: 150 μL of EDTA blood, 75 μL of lysis buffer, and 15 μL of proteinase K. To extract RNA, we used the KingFisher Pure Viral NA Kit (Thermo Fisher Scientific) with the following modifications to volume requirements: 150 μL of EDTA blood, 161 μL of lysis buffer, 4 μL of carrier RNA, 40 μL of proteinase K, and 15 μL of exogenous internal control MS2 (final concentration 1×10^4 copies/μL). Purified DNA and RNA elutes from each specimen were collected from the elution plate and made into two 50-μL aliquots for working (–20°C) and storage (−80°C) stocks.

Control Specimens

As positive controls, we used human DNA; MS2 (Roche Applied Sciences, Laval, QC, Canada); *Plasmodium falciparum* and *P. vivax* small subunit rRNA DNA clones (MRA-177 and MRA-178, MR4; ATCC, Manassas VA, USA); CHIKV MBC099 AmpliRun PCR Controls (Phoenix Airmid Biomedical, Oakville, ON, Canada). We also used previously characterized in-house, positive controls (i.e., herpes simplex virus [HSV]-1 and HSV-2, cytomegalovirus [CMV], Epstein-Barr virus [EBV], DENV serotypes 1–4 [DENV-1–4], and hepatitis A virus [HAV]).

Detection of Viral DNA by Real-Time PCR

We used real-time PCR with previously published primers and probes (Table 1) to test all specimens for viral DNA. A total volume of 25 μL was used; the volume included 12.5 μL of TaqMan Universal MasterMix (Life Technologies, Carlsbad, CA, USA) and 5 μL of template DNA for the *Plasmodium* genus and B2MG assay as well as 3 μL of template DNA for HSV-1, HSV-2, EBV, and CMV assays. All reactions were performed on the Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific), using the following conditions: 2 min at 50°C, 10 min at 95°C, 45 cycles of 15 sec at 95°C, and 1 min at 60°C, according to the manufacturer's protocol. A specimen with a cycle threshold (C_t) of <40 in the presence of a logarithmic amplification curve was considered positive for a viral pathogen.

Detection of Viral RNA by Real-Time Reverse Transcription PCR (RT-PCR)

To test specimens for the presence of viral RNA, we used real-time RT-PCR with previously published primers and probes for dengue-MS2, panflavivirus, HAV, and CHIKV (Table 2). We used the reverse complement of ChikR10487-10508 (5′-GGTGTCCAGGCTGAAGACATTG-3′) because the sense sequence was published for the reverse primer in the CHIKV assay (*28*). A total volume of 20 μL was used, including 5 μL of TaqMan Fast Virus 1-Step MasterMix (Life Technologies, Burlington, ON, Canada) and 3 μL of template RNA. We performed all reactions on the Applied Biosystems 7500 Fast Real-Time PCR System according to the manufacturer's protocol, using the following conditions: 5 min at 50°C, 20 sec at 95°C, 45 cycles of 3 sec at 95°C, and 30 sec at 60°C. A specimen with a C_t <40 in the presence of a logarithmic amplification curve was considered positive for a viral pathogen.

Table 1. Primers and probes used to test specimens from ill travelers returning to Canada for viral DNA*

Primer and probe	Sequence, 5′ → 3′	Concentration, nmol/L	Reference
Human B2MG			(*20*)
B2MG fwd primer	TGAGTATGCCTGCCGTGTGA	900	
B2MG rev primer	ACTCATACACAACTTTCAGCAGCTTAC	900	
B2MG probe	HEX-CCATGTGACTTTGTCACAGCCCAAGATAGTT-BHQ1	200	
Plasmodium			(*21*)
Plasmo1 primer	GTTAAGGGAGTGAAGACGATCAGA	200	
Plasmo2 primer	AACCCAAAGACTTTGATTTCTCATAA	200	
Plasmo probe	FAM-ACCGTCGTAATCTTAACCATAAACTATGCCGACTAG-BHQ1	50	
HSV-1 and -2			(*22*)
GbTypF primer	CGCATCAAGACCACCTCCTC	600	
GbTypR primer	GCTCGCACCACGCGA	600	
GbTyp1 probe	VIC-TGGCAACGCGGCCCAAC-TAMRA	200	
GbTyp2probe	FAM-CGGCGATGCGCCCCAG-TAMRA	200	
CMV			(*23*)
CMV IE fwd primer	GACTAGTGTGATGCTGGCCAAG	500	
CMV IE rev primer	GCTACAATAGCCTCTTCCTCATCTG	500	
CMV IE probe	HEX-AGCCTGAGGTTATCAGTGTAATGAAGCGCC-BHQ1	125	
EBV			(*23*)
EBV-BALF fwd primer	CGGAAGCCCTCTGGACTTC	500	
EBV-BALF5 rev primer	CCCTGTTTATCCGATGGAATG	500	
EBV BALF5 probe	FAM-TGTACACGCACGAGAAATGCGCC-BHQ1	125	

*B2MG, β2 microglobulin; CMV, cytomegalovirus; EBV, Epstein-Barr virus; fwd, forward; HSV, herpes simplex virus; rev, reverse.

Table 2. Primers and probes used to test specimens from ill travelers returning to Canada for viral RNA*

Primer and probe	Sequence, 5′→ 3′	Concentration, nmol/L	Reference
Pandengue-MS2			(*23*,*24*)
Den Eili fwd primer	GGACTAGAGGTTAGAGGAGACCCC	500	
Den Eili rev primer	GAGACAGCAGGATCTCTGGTC	500	
Den Eili probe	FAM-AGCATATTGACGCTGGGA-MGB-BHQ1	125	
MS2-TM2 fwd primer	TGCTCGCGGATACCCG	500	
MS2-TM2 rev primer	AACTTGCGTTCTCGAGCGAT	500	
MS2-TM2 probe	Quasar670-ACCTCGGGTTTCCGTCTTGCTCGT-BHQ1	125	
Dengue virus serotyping			(*25*)
DENV-1 fwd primer	CAAAAGGAAGTCGTGCAATA	500	
DENV-1 rev primer	CTGAGTGAATTCTCTCTACTGAACC	500	
DENV-1 probe	FAM-CATGTGGTTGGGAGCACGC-BHQ1	125	
DENV-2 fwd primer	CAGGTTATGGCACTGTCACGAT	500	
DENV-2 rev primre	CCATCTGCAGCAACACCATCTC	500	
DENV-2 probe	HEX-CTCTCCGAGAACAGGCCTCGACTTCAA-BHQ-1	125	
DENV-3 fwd primer	GGACTGGACACACGCACTCA	500	
DENV-3 rev primer	CATGTCTCTACCTTCTCGACTTGTCT	500	
DENV-3 probe	FAM-ACCTGGATGTCGGCTGAAGGAGCTTG-BHQ1	125	
DENV-4 fwd primer	TTGTCCTAATGATGCTGGTCG	500	
DENV-4 rev primer	TCCACCTGAGACTCCTTCCA	500	
DENV-4 probe	HEX-TTCCTACTCCTACGCATCGCATTCCG-BHQ1	125	
Panflavivirus			(*26*)
Flavi all S (sense)	TACAACATgATggggAARAgAgARAA	500	
Flavi all AS2 (antisense)	gTgTCCCAgCCNgCKgTgTCATCWgC	500	
DENV-4F	TACAACATgATgggRAAACgTgAGAA	500	
Flavi all probe 1	FAM-AARggHAgYMgNgCCA+TH+T+g+g+T-BBQ†	100	
Flavi probe 3a	FAM-CCgTgCCATATggTATATgTggCTgggAgC-BBQ†	100	
Flavi probe 3b	FAM-TTTCTggAATTTgAAgCCCTgggTTT-BBQc	100	
Hepatitis A virus			(*27*); vital SOP‡
HAV 68	TCACCGCCGTTTGCC	500	
HAV240	GGAGAGCCCTGGAAGAAAG	500	
HAV150 probe	FAM-CCTGAACCTGCAGGAATTAA-MGB-NFQ	125	
Chikungunya virus			(*28*)
ChikF10378–10398	GCATCAGCTAAGCTCCGGGTC	500	
ChikR 10487–10508¶	GGTGTCCAGGCTGAAGACATTG	500	
Chik Pongsiri	HEX-ATGCAAACGGCGACCATGCCGTCA-VIC	125	

*The primers and probes were used with the Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). fwd, forward; rev, reverse; SOP, standard operating procedure.
†Locked nucleic acid bases.
‡Vital Standard Operating Procedure modified from Costafreda et al. (*27*).
¶ChikR10487-10508 reverse complement used from published sequence.

Dengue and Panflavivirus Real-Time RT-PCR Assays

A total of 5 μL of real-time RT-PCR product with positive signal (i.e., logarithmic amplification curve) from the DENV or panflavivirus assay were loaded onto a 1% agarose gel and visualized under UV light for any bands. These samples were then used for subsequent sequencing. Using sequence alignment and BLAST searching, we verified that the panflavivirus primers and probes should detect the following viruses, if present in sufficient quantities in a specimen: DENV-1–4; yellow fever (strains 17D, Asibi, Brazil, and Ivory Coast); West Nile virus (strains from Uganda and Israel); tickborne encephalitis virus (strains k23 and Louping ill); Japanese, St. Louis, and Murray Valley encephalitis viruses; and Wesselsbron, Zika, Yaounde, and Nounane viruses.

Dengue Serotype Determination by Duplex Real-Time RT-PCR Assays

Before sequencing pan-DENV– and panflavivirus-positive samples, we performed second confirmatory assays on all positive specimens to detect all 4 DENV serotypes; the assays consisted of 2 duplex real-time RT-PCRs targeting DENV-1/2 and DENV-3/4, respectively (Table 2). Two separate duplex reactions were performed with a volume of 25 μL. The first reaction used 12.5 μL of QuantiTect Probe RT-PCR MasterMix (QIAGEN, Germantown, MD, USA); 500 nmol/L primers DENV-1JF, DENV-1JR, DENV-2JF, and DENV-2JR; 125 nmol/L probes 1Jpr, DENV-2Jpr, DENV-3Jpr, and DENV-4Jpr; and 5 μL of template RNA. The second reaction used 12.5 μL of QuantiTect Probe RT-PCR MasterMix; 500 nmol/L primers DENV-3JF, DENV-3JR, DENV-4JF, and DENV-4JR; 125 nmol/L probes DENV-3Jpr and DENV-4Jpr; and 5 μL of template RNA. We used the Applied Biosystems 7500 Fast Real-Time PCR System with the following cycling conditions: 30 min at 50°C, 15 min at 95°C, 45 cycles of 15 sec at 94°C, and 1 min at 60°C.

Sequencing and Analysis

We performed Sanger sequencing reactions using the Big Dye v3.1 Cycle Sequencing Kit (Life Technologies,

Carlsbad, CA, USA). For each cycle sequencing reaction, we used a 20-μL volume, including 1 μL of PCR product, 2 μL of Big Dye, 3 μL of buffer, and 2 μL of 10 μmol/L primer. We used a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions and under the following cycling conditions: 1 min at 96°C, 25 cycles of 10 sec at 96°C, 5 sec at 50°C, and 4 min at 60°C. We then cleaned the product, using the DyeEx 2.0 Spin Kit (QIAGEN), and placed it into a speed vacuum for 35 min, including 10 min of heat. Next, we added 20 μL of Hi-Di Formamide (Thermo Fisher Scientific) to each sample and performed sequencing using the ABI 3730xl DNA Analyzer (Life Technologies). We used ABI Sequencing Analysis Software 5.2 program (Life Technologies) to standardize data and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) to analyze sequences, which were subsequently BLAST-searched to identify the pathogen to the genus and species levels.

Statistical Analyses

We calculated descriptive statistics (mean, median, range) for all continuous variables and compared differences between groups by using Student *t*-test or analysis of variance (ANOVA), or, in the case of nonnormal distribution, the Mann-Whitney rank-sum test or 1-way ANOVA on ranks. We quantified categorical variables by proportions and used Yates-corrected χ^2 analysis to compare differences. We used GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) to perform all statistical computations. Significance was set at $p<0.05$.

Results

We routinely screened 1,592 specimens for malaria during the enrollment periods of May 2006–April 2007 and February 2013–March 2014. Of those specimens, 165 (10.4%) were positive for *P. falciparum*, 93 (5.8%) for *P. vivax*, 20 (1.3%) for *P. ovale*, and 5 (0.3%) for *P. malariae*; 5 (0.3%) specimens had mixed *Plasmodium* infections. We subjected the remaining 1,308 malaria-negative specimens to the real-time PCR algorithm as described in the Methods to look for RNA- and DNA-based travel-acquired viruses. We did not assess the 165 malaria-positive specimens for co-infection with viral pathogens.

All assays detected control samples that had been confirmed positive. The dengue serotype–specific assay confirmed all types of DENV (DENV-1–4) without cross-reactivity among serotypes. Of the 1,308 malaria-negative specimens that were examined for multiple viral targets, only 1,304 could be tested as 4 were invalid (i.e., the internal reaction control β_2 microglobulin could not be detected). A total of 262 (20.1%) of 1,304 specimens were positive (C_t <40) for at least 1 viral pathogen, 293 (22.5%) were positive for any detectable C_t and logarithmic amplification curve, and 31 (2.4%) had a C_t >40 with logarithmic amplification. DNA-based real-time PCR assays detected specimens positive for HSV-1 (n = 7, 0.54%), HSV-2 (n = 14, 1.1%), CMV (n = 4, 0.3%), and EBV (n = 194, 14.9%). RNA-based real-time RT-PCR assays detected CHIKV in 5 specimens (0.4%) and HAV in 12 (0.9%). real-time RT-PCR and subsequent Sanger sequencing identified DENV-positive specimens (n = 33, 2.5%) and untypeable flavivirus (n = 2, 0.15%). DENV subtyping by serotype-specific real-time RT-PCR and Sanger sequencing detected specimens positive for DENV-1 (n = 12, 0.92%), DENV-2 (n = 3, 0.23%), DENV-3 (n = 9, 0.69%), DENV-4 (n = 3, 0.23%), and untypeable DENV (n = 6, 0.46%). Nine (0.7%) specimens had mixed viral infections: EBV and DENV-1 (3 specimens), EBV and HAV (2 specimens), EBV and HSV-1 (2 specimens), DENV-1 and CHIKV (1 specimen), and CMV and HAV (1 specimen).

Demographic Correlates of Detected Viral Pathogens

Of 1,304 malaria-negative EDTA-containing specimens tested for viral pathogens, 656 (50.3%) were from men and 621 (47.6%) from women; patient sex was unknown for 23 (2.1%) specimens. The distribution of cases between male and female patients was roughly the same: of the 262 specimens that were positive for at least 1 of the tested viruses, 132 (50.4%) were from male and 124 (47.3%) from female patients ($p = 0.10$). The lack of sex bias was observed across each assay. The average age of febrile returned travelers was 39.7 ± 23.1 years (median 39.1, range 10 months–90.6 years). Mean ages of patients infected with different viral pathogens were 23.0 ± 24.5 years for HSV-1, 36.5 ± 24.4 years for HSV-2, 41.6 ± 24.2 years for EBV, 32.3 ± 17.5 years for CMV, 37.6 ± 14.9 years for DENV, 36.7 ± 17.1 years for any flavivirus, 41.8 ± 10.2 years for CHIKV; and 27.5 ± 20.5 years for HAV ($p = 0.17$). Travel history was noted for 129 (49.2%) of the patients from whom the 262 specimens with at least 1 viral pathogen were derived (Table 3).

Discussion

We document common viral etiologies of fever in returned travelers by highly sensitive real-time PCR assays in >20% of malaria-negative specimens collected prospectively at a reference laboratory. At present, real-time PCR remains the best method for detecting viral pathogens causing acute illness, especially when low virus load is present in peripheral blood (*29*). In addition to its high sensitivity (and low limit of detection), real-time PCR offers the advantage of a fast turnaround time, operator independence, and the potential for high-throughput screening, testing, and detection; thus, it is a favored diagnostic tool, especially for detecting viruses that lead to transient and early viremia.

Table 3. Region of travel for patients from Canada who had viral pathogen–positive specimens after returning ill from travel, May 2006–April 2007 and February 2013–March 2014*

Viral pathogen	Total no. pathogens detected	No. (%) specimens with patient travel history noted	Region of travel, no. patients
HSV			
HSV-1	7	4 (57.1)	Afghanistan, 1; Caribbean, 1; Haiti, 1; Hawaii, 1
HSV-2	14	6 (42.9)	Dominican Republic, 3; India, 1; Sri Lanka, 1; Venezuela, 1
EBV	194	96 (47.7)	Indian subcontinent, 34; Caribbean, 21; Africa, 17; South America, 7; Central America, 5; Southeast Asia, 5; North America, 4; Middle East, 3
CMV	4	2 (50.0)	Pakistan, 1; South America, 1
Chikungunya virus	5	3 (60.0)	Barbados, 1; Philippines, 1; South Africa, 1
Dengue virus, serotype	33	20 (60.6)	
DENV-1	12	10 (83.3)	Barbados, 4; Cambodia, 1; Caribbean, 1; Dominican Republic, 1; India, 1; Sri Lanka, 1; Trinidad, 1
DENV-2	3	3 (100)	Bangladesh, 1; Cuba, 1; Tanzania, 1
DENV-3	9	2 (22.2)	Indonesia, 1; Philippines/Vietnam, 1
DENV-4	3	2 (66.7)	Vietnam, 2
Untypeable dengue	6	3 (50.0)	Guyana, 1; Malaysia, 1; Yemen, 1
Untypeable flavivirus	2	1 (50.0)	Barbados, 1
HAV	12	4 (33.3)	Pakistan, 3; Bangladesh, 1

*Data are for 129 (49.2%) of 262 EDTA-containing specimens. CMV, cytomegalovirus; DENV-1–4, dengue virus type 1–4; EBV, Epstein-Barr virus; HAV, hepatitis A virus; HSV-1 and 2, herpes simplex 1 and 2 virus.

The lack of an etiologic diagnosis for up to 25% of febrile returning travelers is probably a result of insensitive diagnostic techniques (e.g., blood culture for detection of *Salmonella* Typhi), serologic testing that is performed outside the optimal acute and convalescent-phase window, and etiologies for which no readily available diagnostic test exists (e.g., novel and emerging flaviviruses, such as Zika virus). In addition, most testing done for the investigation of posttravel fever relies on single-pathogen detection assays, meaning that if a clinician fails to consider a particular virus in the differential diagnosis, testing for that virus will not automatically occur.

We noted high positivity rates for human herpesviruses in this study. Ninety-five percent of the human population is exposed to EBV and CMV by the age of 40, and the infection is often asymptomatic (*30*). Infection with HSV-1 and HSV-2 is not as common, however; the prevalence of infection within a population is 70%–100% (HSV-1) and 6%–50% (HSV-2) by 40 years of age (*30*). These herpesviruses replicate both lytically and latently; most infections are latent in the nervous system and lymphoid tissue. The herpesvirus positivity rates in our study ranged from 0.3% (HSV-1 and CMV) to 14.8% (EBV), numbers that are too low to reflect baseline prevalence rates in the general population and isolated latent infection. Rather, their detection probably reflects acquisition during travel or reactivation, either at home or abroad, in the setting of another antecedent illness. Another possibility would be primary infection before travel, with manifestation during or after travel. Indeed, all but 1 of 9 co-infections detected in this study involved at least 1 human herpesvirus, suggesting that primary acquisition of 1 virus precipitated secondary reactivation of another. Human herpesviruses can all cause a mononucleosis-like syndrome characterized by fever, malaise, lymphadenopathy, and mild biochemical hepatitis, and this nonspecific syndrome has been found to occur in up to a quarter of febrile returned travelers (*2*). Further delineation of the role of human herpesviruses in imported fever warrants prospective investigation with clinical linkage.

Flaviviruses include >70 different arthropodborne species, such as DENV, yellow fever virus, West Nile virus, Japanese encephalitis virus, and tickborne encephalitis virus, which are differentiated into 3 virus groups: mosquitoborne, tickborne, and unknown vector (*26*). Next to EBV, DENV was the most frequently detected viral pathogen in this study. Although DENV is endemic to many countries of Africa, Southeast Asia, and the Western Pacific region, where 50–100 million cases are reported each year (*26*), it causes disease that is increasingly imported from popular tourist destinations in the Americas, including the Caribbean. Thirty-percent of patients in this study with DENV-positive specimens had noted travel history to the Caribbean, and DENV-1 was particularly represented among these cases (7/12 cases). Our finding of only 2 undetermined flaviviruses is curious, given the broad *Flaviviridae* coverage of the selected primer and probe set (*26*). We cannot exclude the possibility of assay failure, but the possibility also exists of an importation of rare or novel flaviviruses acquired during travel.

CHIKV (family *Togaviridae*, genus *Alphavirus*) is also a mosquitoborne virus; the vectors, *Aedes albopictus* and *A. aegypti*, are known to carry DENV (*31*). Although dengue fever and chikungunya fever may have an identical early clinical manifestation, it is critical that the viral pathogen causing illness be identified in order to stratify patients by risk for complications arising from infection,

including chronic arthropathy in the case of CHIKV infection and severe disease in the case of DENV infection. Serologically differentiating DENV from CHIKV may be challenging, given assay performance characteristics, timing of serologic testing, and known cross-reactivity of DENV IgG with that of other flaviviruses, including yellow fever vaccine virus (*31*). In addition, in the case of CHIKV, reliance on assays based on IgM only may lead to underdiagnosis during the convalescent phase of infection (*11*). We assume that enrollment of specimens further into 2014 and 2015 would have translated into much higher numbers of cases, given the maturation of the CHIKV outbreak in the Caribbean and Latin America.

Hepatitis A is one of the most common vaccine-preventable diseases, but the level of pretravel vaccination continues to be suboptimal among persons visiting HAV-endemic countries (*32*). In a study conducted with travelers from Sweden, the risk for travel-associated HAV infection was 0.67 cases per 100,000 travelers (*32*). The risk for infection is high for travelers to South America, Africa, and the Indian subcontinent; in our study, all HAV-positive patients with travel history noted had traveled to Bangladesh or Pakistan. That 0.9% of specimens were positive for HAV in this study underscores the remaining gap in pretravel vaccination.

Our analysis had several limitations. First, we lacked clinical linkage of specimens; thus, in each case, the exact clinical syndrome that prompted malaria screening is unknown. Given that malaria is not endemic to Canada and that persons who warrant malaria screening are likely unwell, we were reasonably confident that all included specimens would be from ill persons with a travel history. Second, we limited our viral detection algorithm to those viruses or virus families that are known to commonly cause fever in returned travelers. Therefore, our results probably represent an underestimate of virus burden in this cohort of specimens. For instance, we did not look for HIV, a virus that is an infrequent cause of fever in returned travelers, nor did we search for the agents of viral hemorrhagic fever, which, before the 2013–2015 Ebola virus crisis in western Africa, were exported less than a handful of times by travelers. We also did not search for influenza or other predominantly respiratory viruses due to the absence of a true viremic stage in the infection process, although these agents are well recognized to cause fever in returned travelers. Third, we relied exclusively on the specimen requisition forms for acquiring travel histories for patients, and forms for less than half of the patients included this information. The lack of travel histories may have biased our interpretation of the geographic representation of detected pathogens. Fourth, we only performed our diagnostic assays on EDTA-containing blood, the sensitivity of which would be affected by the level of viremia and the timing of initial malaria diagnostics. For instance, viremia in CHIKV and DENV infection is short-lived, so the prevalence of DENV and CHIKV might have been higher had serologic evaluations been used. Fifth, our reported rates of viral illnesses could represent an underestimate if the initial malaria diagnostic specimen came from a patient who was afebrile and without risk factors for travel-acquired illness. We believe that this possibility is low because malaria testing is not routine and, if anything, is underordered in areas where malaria is not endemic. Last, as mentioned, we were unable to differentiate primary infection from reactivation disease in the specimens positive for human herpesviruses.

We documented infections with common and emerging viral pathogens, including 12 cases of acute hepatitis A, which is vaccine preventable, in >20% of malaria-negative specimens from travelers who were ill when they returned to Canada. A substantial diagnostic gap remained in this group of specimens from febrile returned travelers, despite application of highly sensitive and family-level real-time PCR assays. Even in the setting of positive flavivirus and DENV real-time PCR, some viruses remained untypeable. Further elucidation of the specific etiology of fever in returned travelers should be undertaken with increasingly sophisticated diagnostics, such as next-generation sequencing, to better understand the full spectrum of pathogens causing this potentially life-threatening syndrome.

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Expanded Geographic Distribution and Clinical Characteristics of *Ehrlichia ewingii* Infections, United States

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Ehrlichiosis is a bacterial zoonosis, spread through the bites of infected ticks, that is most commonly caused in the United States by infection with the bacterium *Ehrlichia chaffeensis*. We retrospectively reviewed samples from an 18-month study of ehrlichiosis in the United States and found that *E. ewingii* was present in 10 (9.2%) of 109 case-patients with ehrlichiosis, a higher rate of infection with this species than had previously been reported. Two patients resided in New Jersey and Indiana, where cases have not been reported. All patients with available case histories recovered. Our study suggests a higher prevalence and wider geographic distribution of *E. ewingii* in the United States than previous reports have indicated.

Ehrlichiosis is a bacterial zoonosis spread through the bites of infected ticks. Three species have been identified as causes of ehrlichiosis within the United States: *Ehrlichia chaffeensis*, *E. ewingii*, and an *E. muris*–like pathogen (*1*). Anaplasmosis is a disease with an overlapping clinical syndrome caused by the closely related organism *Anaplasma phagocytophilum* (*2*). *E. chaffeensis* and *E. ewingii* are spread by *Amblyomma americanum* ticks, which are found widely distributed across the eastern and southeastern United States. In contrast, *A. phagocytophilum* is primarily transmitted by *Ixodes scapularis* ticks in the northeastern and upper Midwest regions. *Ix. scapularis* ticks are the possible vector of the *E. muris*–like pathogen. This organism has been detected in *Ix. scapularis* ticks in Minnesota and Wisconsin, where 69 cases of human infection with the *E. muris*–like pathogen have been reported (*1*,*3*).

E. chaffeensis is the most common cause of human ehrlichiosis; 1,518 were cases reported in 2013 mainly in south-central, southeastern, and mid-Atlantic states (*4*). Signs and symptoms for this disease include fever, chills, headache, myalgia, malaise, thrombocytopenia, leukopenia, and increased levels of liver enzymes (*5*). Severe infections can occur (case-fatality rate ≈1%) and are a concern in immunocompromised patients (*6*). *E. ewingii* is a less common human pathogen; it accounted for 31 cases of ehrlichiosis in 2013 (*4*). Clinical disease caused by *E. ewingii* has not yet been well characterized.

E. ewingii is prevalent in dogs and white-tailed deer throughout the central and southeastern United States, and reported human infections have increased since the disease became reportable in 2008 (*4*,*7*,*8*). The first human cases of infections were reported in 4 patients in Missouri in 1999, three of whom were immunosuppressed (*9*). *E. ewingii* was subsequently reported in 4 symptomatic patients from Missouri, Oklahoma, and Tennessee, all of whom were co-infected with HIV (*10*). One case of *E. ewingii* infection that was likely acquired through platelet transfusion from an asymptomatic donor with tick exposure has been reported (*11*). Most recently, *E. ewingii* was detected in the peripheral blood and bone marrow of a symptomatic 65-year-old woman from Arkansas (*12*). The most consistent clinical findings in these patients were fever and thrombocytopenia. No deaths have been reported.

Ehrlichia spp. are obligate intracellular organisms, and morulae (bacterial clusters within cytoplasmic vacuoles) are visualized on peripheral blood films. However, detection of morulae in monocytes (for *E. chaffeensis*) or granulocytes (for *E. ewingii*) has limited sensitivity (*13*). Serologic testing for *E. chaffeensis* can be performed but is insensitive during the acute phase of illness and has limited specificity (*5*,*9*). Specific serologic testing for *E. ewingii*

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is not available. The extent of cross-reactivity of serologic tests for *E. chaffeensis* with *E. ewingii* is unclear, but use of serologic testing alone might contribute to underreporting of infection with *E. ewingii*. Thus, diagnosis of infection with *E. ewingii* is reliant on species-specific molecular testing. The purpose of this study was to determine the geographic distribution and clinical characteristics of PCR-confirmed *E. ewingii* infections in the United States.

Methods

We retrospectively reviewed results of 18 months (May 2013–November 2014) of testing for human ehrlichiosis by real-time PCR. All samples submitted to Associated Regional and University Pathologists Laboratories (Salt Lake City, UT, USA) for *Ehrlichia* and *Anaplasma* species by real-time PCR were included in the analysis. PCR-positivity rates for *Ehrlichia* spp. were calculated on the basis of results for individual patients.

We used a real-time PCR for *Ehrlichia* and *Anaplasma* species that detects *E. chaffeensis, E. muris*–like pathogen, *E. ewingii*, and *E. canis* (without differentiating *E. ewingii* and *E. canis*) and *A. phagocytophilum*. In brief, nucleic acids extracted from whole blood specimens by using the Chemagic MSM I Automated Extraction Platform and the Chemagen Blood Extraction Kit (Perkin Elmer, Waltham, MA, USA) were amplified by using primers specific for 16S rRNA gene and *Ehrlichia* and *Anaplasma* species–specific probes for identification (Table 1). The reaction was prepared by using a 5× custom real-time Master Mix and 4 mmol/L $MgCl_2$ (Promega, Madison, WI, USA) with the following amplification parameters: 50.0°C for 2 min; denaturation at 95.0°C for 2 min; and 50 cycles at 95.0°C for 5 s, 56.0°C for 20 s, and 76.0°C for 20 s on the Rotor-Gene Q apparatus (QIAGEN, Hilden Germany). Melting curve analysis was performed at 95.0°C for 15 s and then from 45°C through 75°C at 1.0°C/step at 5 s/step with continuous fluorescence acquisition.

We retained samples positive for *E. ewingii/E. canis* from the initial tests for further analysis for this study. Nucleic acids were extracted from these residual blood specimens, and another real-time PCR specific for a region within the 16S rRNA gene was performed to differentiate *E. ewingii* from *E. canis*. We contacted healthcare providers for *E. ewingii*–positive patients to collect case histories. The study was approved by the University of Utah Institutional Review Board (no. 76713).

Results

Of 4,177 patients from 41 states who had samples submitted to Associated Regional and University Pathologists Laboratories for detection of *Ehrlichia* and *Anaplasma* species by real-time PCR during an 18-month study period, 99 (2.4%) were positive for *E. chaffeensis*, 10 (0.2%) for *E. ewingii/E. canis*, and 0 for *E. muris*-like pathogen. A total of 179 (4.3%) patients were positive for *A. phagocytophilium*. Positivity rates were calculated by state (Table 2).

All 10 *E. ewingii/E. canis*–positive cases were subsequently identified as *E. ewingii*, accounting for 10 (9.2%) of 109 ehrlichiosis cases during the study period. *E. ewingii*–positive samples were from 9 men and 1 woman (median age 58 years, range 24–74 years). The samples were from Missouri (n = 4), Indiana (n = 3), Arkansas (n = 1), New Jersey (n = 1), and New York (n = 1) and were collected in June (n = 1), July (n = 3), August (n = 4), and September (n = 2). Case histories and laboratory results were obtained for 5 patients (Table 3).

Case-patient 1 was 24-year-old man (landscaper) from New Jersey who had nausea, vomiting, and fevers in August 2014. The patient had no known tick bites and no major underlying concurrent illnesses. At the time of presentation, his temperature was 102°F; results of a physical examination were otherwise unremarkable. Laboratory testing showed thrombocytopenia, and blood cultures obtained at presentation were negative for bacterial growth. Serologic testing for Lyme disease by ELISA showed antibodies against *Borrelia burgdorferi*. Western blot showed a positive result for IgM and a negative result for IgG. Serologic testing for *Ehrlichia* spp. was not performed. The patient received a 2-week course of doxycycline, and his symptoms resolved.

Case-patient 2 was a 38-year-old man from Missouri who had a history of headache and fever for 10 days in August 2014. The patient had no known tick exposure, and his medical history was unremarkable. At the time of presentation, his temperature was 102°F. He had an eczematous rash on his left upper leg and increased levels of liver

Table 1. Primers and probes used in real-time PCR for *Ehrlichia* and *Anaplasma* species, United States*

Name	Sequence, 5′ → 3′	Concentration, nmol/L	Species detected
Primer 1	GCATTACTCACCCGTCTGCCACT	250	–
Primer 2	CAAGCCTAACACATGCAAGTCGAACG	1,000	–
Probe 1	MGB-FAM-AGGT†T†ATAA†GCA†ATTGTCC-EDQ	200	*E. chaffenisis*
Probe 2	MGB-(AP642)-AAGCTATA†GGCA†GT†TA†TCC-EDQ	200	*E. muris*–like agent
Probe 3	MGB-(AP593)-GGCTATA†A†ATA†A†TTGT†CCG-EDQ	200	*E. canis*
Probe 4	MGB-(AP593)-CTATTTA†GGA†A†TTGT†T†C-EDQ	200	*E. ewingii*
Probe 5	MGB-(AP525)-AAAGAAT†A†A†TCCGTTCG-EDQ	200	*A. phagocytophilum*

*–, these primers are specific for 5 organisms; MGB, minor groove binder; FAM, 6-carboxyfluorescein; EDQ, Eclipse Dark Quencher (Epoch Biosciences Inc., Bothell, WA, USA).
†Use of Super A, T, or G (Epoch Biosciences Inc.).

Table 2. Results for patients tested for infection with *Ehrlichia* and *Anaplasma* species by real-time PCR, United States

State*	No. tested	No. (%) positive		
		E. chaffeensis	*E. canis/ewingii*	*A. phagocytophilum*
Arkansas	3	0 (0)	1 (33.3)	0 (0)
Colorado	3	1 (33.3)	0 (0)	0 (0)
Connecticut	23	0 (0)	0 (0)	3 (13)
Georgia	7	1 (14.2)	0 (0)	0 (0)
Illinois	70	4 (5.7)	0 (0)	0 (0)
Indiana	263	25 (9.5)	3 (1.1)	0 (0)
Iowa	4	1 (25)	0 (0)	0 (0)
Kansas	52	10 (19.2)	0 (0)	0 (0)
Kentucky	26	8 (30.8)	0 (0)	0 (0)
Louisiana	11	1 (9.1)	0 (0)	0 (0)
Maine	375	0 (0)	0 (0)	17 (4.5)
Massachusetts	526	0 (0)	0 (0)	40 (7.6)
Michigan	3	1 (33.3)	0 (0)	0 (0)
Minnesota	930	0 (0)	0 (0)	44 (4.7)
Missouri	94	15 (15.9)	4 (4.3)	0 (0)
Nebraska	69	8 (11.6)	0 (0)	1 (1.4)
New Hampshire	708	1 (0.1)	0 (0)	51 (7.2)
New Jersey	202	7 (3.5)	1 (0.5)	5 (2.5)
New York	283	3 (1.1)	1 (0.4)	6 (2.1)
Pennsylvania	72	2 (2.8)	0 (0)	1 (1.4)
Tennessee	150	6 (4)	0 (0)	0 (0)
Texas	52	2 (3.8)	0 (0)	1 (1.9)
Utah	16	1 (6.3)	0 (0)	0 (0)
Virginia	28	0 (0)	0 (0)	1 (3.6)
Washington	24	2 (8.3)	0 (0)	0 (0)
Wisconsin	68	0 (0)	0 (0)	9 (13.2)

*States for which all patents showed negative results are Alabama (n = 2), Arizona (n = 11), California (n = 19), Florida (n = 9), Hawaii (n = 1), Idaho (n = 1), Montana (n = 1), Nevada (n = 3), North Carolina (n = 6), North Dakota (n = 2), Ohio (n = 25), Oregon (n = 8), Rhode Island (n = 3), South Dakota (n = 21), and Wyoming (n = 3).

enzymes. Results of serologic testing for *E. chaffeensis* were negative. The patient received a 1-week course of doxycycline, and his symptoms resolved.

Case-patient 3 was a 73-year-old man from Indiana who had subjective fevers, mild left lower abdominal pain, myalgia, and malaise. The patient reported a tick bite on his abdomen 2–3 weeks before presentation. His medical history included type 1 diabetes, hypertension, and stroke. Results of a physical examination were unremarkable. He was afebrile at presentation but had leukopenia and thrombocytopenia. Results of serologic testing for Lyme disease (total antibodies against *B. burgdorferi* by ELISA) were negative. Serologic testing for *Ehrlichia* spp. was not performed. The patient received a 10-day course of doxycycline, and his symptoms resolved.

Case-patient 4 was a 74-year-old man from Arkansas who had malaise, arthralgia, fever, and nonbloody diarrhea in for 2 weeks in July 2014. The patient reported 2 tick bites 2 weeks before presentation. His medical history included diffuse large B-cell lymphoma (treated with gemcitabine), lung cancer (treated with a right lobectomy), chronic obstructive pulmonary disease that required supplemental oxygen, coronary artery disease, and hypothyroidism. At presentation, he had hypotension (79/46 mm Hg), tachycardia (142 beats/min), and a fever (temperature 100.1°F). Laboratory testing showed leukopenia, thrombocytopenia, and morulae in neutrophils on a peripheral blood smear. Results of serologic testing for *E. chaffeensis* were negative. The patient received a 3-week course of doxycycline and showed clinical improvement; however, he died 1 month later of unrelated bacterial sepsis.

Case-patient 5 was a 73-year-old man from Missouri who had headache, fever, and nausea in August 2014. The patient reported a tick bite ≈2 months before presentation. His medical history included bladder cancer (treated with immunotherapy), a melanoma on his right arm (treated with surgical excision), type 1 diabetes, and hypothyroidism. He had leukopenia, thrombocytopenia, and increased levels of liver enzymes. Blood cultures at presentation and serologic results for *E. chaffeensis* were negative.

Table 3. Associated laboratory findings for 5 patients infected with *Ehrlichia ewingii*, United States

Laboratory finding	Patient				
	1	2	3	4	5
Minimum leukocyte count, × 10^3 cells/μL	3.5	4.5	2.7	2.6	2.8
Minimum platelet count, × 10^3/μL	128	179	102	92	100
Maximum aspartate aminotransferase level, U/L	24	115	38	28	91
Maximum alanine aminotransferase level, U/L	26	279	38	28	78
Maximum alkaline phosphatase level, U/L	77	90	85	87	146

The patient received a 10-day course of doxycycline, and his symptoms resolved.

Discussion

In our study, *E. ewingii* accounted for 10 (9.2%) of 109 cases of *Ehrlichia* spp. infections, compared with only 31 (2.0%) of 1,518 reported human ehrlichiosis cases in the United States (*4*). Underreporting might be caused by successful empirical treatment without etiologic diagnosis; missed *E. ewingii* infections by serologic tests for *E. chaffeensis* in the acute phase of illness (case-patients 2, 4, and 5); and limited availability of molecular tests. Even with molecular testing, cases might be missed because of non-optimal timing of specimen collection and limited sensitivity of the assay, such as with testing after resolution of bacteremia.

Human infections with *E. ewingii* naturally acquired have been reported in Arkansas, Missouri, Oklahoma, and Tennessee (*9,10,12*). We now describe cases of infection in Indiana and New Jersey. The location of these cases is consistent with the known range of the vector (*A. americanum* ticks). *E. ewingii* has previously been reported in *A. americanum* ticks collected in New Jersey (*14*).

The 5 case-patients reported in this study had symptoms of classical ehrlichiosis, including fever, myalgia, malaise, and headache. Thrombocytopenia and leukopenia were the most consistent associated laboratory findings. All patients improved after treatment with doxycycline treatment, despite in some instances, major underlying disease. *E. ewingii* should be considered as an etiologic agent of tickborne febrile illness in the central and eastern United States that may be missed by serologic testing.

Dr. Harris is a clinical pathologist in The Mount Sinai Health System and at The Icahn School of Medicine at Mount Sinai, New York, New York. Her primary research interest is optimizing use of microbiological testing services.

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Molecular Characterization of Canine Rabies Virus, Mali, 2006–2013

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We genetically characterized 32 canine rabies viruses isolated in Mali during 2006–2013 and identified 3 subgroups that belonged to the Africa 2 lineage. We also detected subgroup F rabies virus. This information should be useful for development of mass vaccination campaigns for dogs and eventual large-scale control programs in this country.

Rabies causes an estimated 70,000 human deaths annually worldwide, and >99% occur in developing countries, of which ≈43% occur in Africa, where rabies virus circulates in the dog population (*1*). A person bitten by a rabid dog, if not given postexposure prophylaxis, has an ≈5% (if bitten on the hand) to 70% (if bitten on the face) probability of showing development of clinical rabies (*2*). However, postexposure prophylaxis is often unavailable or unaffordable in many developing countries.

Numerous infectious diseases, including tuberculosis, malaria, dengue fever, and rabies, are present in Mali. The domestic dog is the major reservoir and vector of rabies in this country. Although disease surveillance is insufficient throughout Mali, the level of underreporting of rabies cases is unknown. Animal and human cases are recorded mainly in urban and suburban areas. Surveillance data reflect rabies mainly in Bamako (the capital of Mali; population 1.8 million), where rabies diagnostic testing is available.

A standard procedure is in place in Bamako for reporting of an animal bite. The bitten person should immediately contact the Division of Epidemiology, Prevention and Control of Diseases, which is part of the National Directorate of Health. Persons with suspected cases of rabies are referred to a specialized clinic (Lazaret Clinic) in Bamako. Dog owners are requested by the Division of Epidemiology, Prevention and Control of Diseases to bring their dogs to a veterinary clinic for a 15-day quarantine. Rabies diagnosis of suspect animals is made by the Central Veterinary Laboratory (CVL) in Bamako. A diagnosis of rabies in humans is based only on results of a clinical examination because of sociocultural reasons (*3*). In other cities in Mali, there are reference health centers, hospitals, and veterinary regional services for diagnosis (*4*).

During 2000–2013, samples from 468 animals showing clinical signs of rabies or to whom humans were exposed were submitted to the CVL for rabies testing by using the fluorescent antibody test (*5*). Of 468 animals analyzed, 447 (435 dogs, 4 cats, 4 cows, and 4 monkeys) showed positive results for rabies. Twenty-eight human cases of rabies were reported during 2007–2009 in Bamako, which indicated an incidence of 3.3 cases/1,000,000 persons/year despite 141 postexposure prophylaxis vaccinations/1,000,000 persons/year (*4*). Assuming a dog:human ratio in Bamako of 1:121, the annual incidence of rabies in dogs is ≈2.24 rabid dogs/1,000 dogs during the past 13 years, which is higher than that observed in N'Djaména, Chad (*6*), which borders Mali.

A total of 306 (45.0%; 95% CI 38%–52%) of 680 dogs were reported as being vaccinated against rabies at least once. However only 59 (19.3%) of the 306 dogs examined had a valid vaccination certificate (*4*).

In Bamako, an average of 1,470 persons are bitten by animals each year, of whom 1,427 (97.1%) are bitten by dogs (*3*). A total of 3,544 (60.3%) of 5,870 bitten persons are young adults, including 1,920 (32.73%) children <10 years of age. Men are bitten more often than women.

Four lineages (Africa 1–4) of rabies virus and several subgroups have been detected in Africa. All lineages include classical rabies virus species and vary by geographic area, virus evolution, and reservoir species (*7*,*8*). The most comprehensive study of western and central African rabies viruses included some isolates from Mali (*7*). The purpose of our study was to obtain more detailed information on genetic characteristics of rabies virus circulating in Mali and to clarify the geographic distribution and transboundary spread of this virus in the canine population in Mali.

The Study

During 2002–2013, a total of 468 specimens were submitted from various regions in Mali to the CVL for rabies diagnosis (Figure 1). Samples were tested by using the

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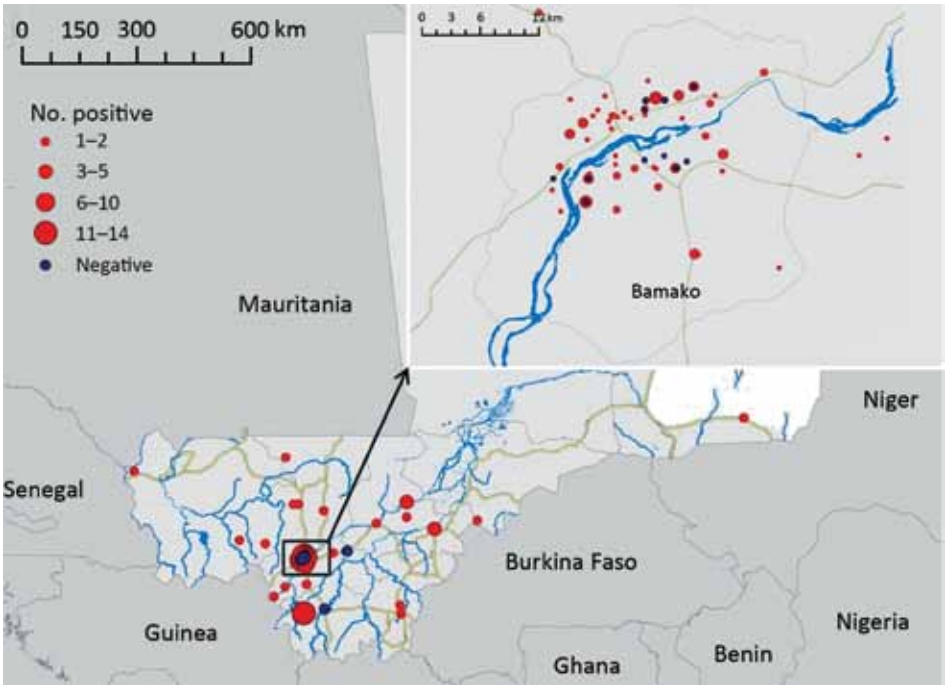

Figure 1. Locations of origin for 100 specimens analyzed in this study (95 with positive results and 5 with negative results) submitted for rabies virus diagnosis, Mali, 2002–2013. Inset shows closer view of the area near the capital city of Bamako.

Table 1. Characteristics of 32 rabies virus samples from dogs, Mali, 2006–2013*

Virus	GenBank accession no.	Sample ID no.	Region	Quantitative RT-PCR C_t	Subgroup of Africa 2 lineage
RV01	KP976113	420/2006	Bamako	28.51	G
RV04	KP976114	345/2007	Bamako	30.19	H
RV05	KP976130	352/2007	Bamako	25.35	G
RV06	KP976125	58/2008	Bamako	24.09	G
RV09	KP976126	146/2008	Bamako	27.51	G
RV10	KP976122	154/2008	Ségou	25.75	G
RV11	KP976124	167/2008	Koulikoro	23.97	G
RV14	NA	259/2008	Bamako	31.59	H
RV15	KP976123	261/2008	Ségou	26.14	G
RV19	NA	530/2008	Bamako	27.85	G
RV20	KP976117	003/2009	Bamako	32.82	H
RV22	NA	69/2009	Bamako	26.15	H
RV27	NA	118/2009	Bamako	32.30	H
RV44	KP976129	587/2009	Bamako	26.22	G
RV50	NA	19/11/2010	Bamako	27.90	G
RV51	NA	42/2010	Bamako	28.59	G
RV56	NA	171/2010	Koulikoro	22.20	G
RV57	KP976121	176/2010	Gao	21.90	F
RV60	NA	221/2010	Bamako	24.60	H
RV67	NA	603/2010	Bamako	21.30	H
RV68	NA	137/2011	Bamako	20.80	H
RV70	KP976119	149/2011	Bamako	21.70	H
RV79	NA	339/2011	Bamako	24.90	G
RV81	KP976127	357/2011	Bamako	34.20	G
RV84	NA	480/2011	Bamako	21.20	G
RV87	KP976116	612/2011	Bamako	22.50	H
RV88	KP976120	628/2011	Koulikoro	21.70	H
RV89	NA	674/2011	Bamako	20.20	H
RV90	KP976118	688/2011	Bamako	30.80	H
RV93	KP976115	223/2012	Bamako	23.20	H
RV95	NA	366/2012	Bamako	21.00	G
RV96	KP976128	100/2013	Bamako	29.00	G

*A fluorescent antibody test was conducted as described by Dean et al. (*5*). For each tested sample, test paper was impregnated with 100 μL of 10% brain suspension and subjected to molecular biological analysis. Of 100 samples tested, 32 showed positive results by this test. A conventional hemi-nested reverse transcription PCR (RT-PCR) was performed with rabies virus primers JW12–JW6 as described (*9*). All samples showed positive results by this test. A quantitative RT-PCR was performed with rabies primers JW12–N165-146 (*10*). This PCR detected ≥100 RNA copies/μL. The coefficient of determination (R^2) was 0.999, the Y intercept was of 36.65, and efficiency was 99%. Samples in bold (n = 15) had duplicate sequences and were not subjected to phylogenetic analysis. ID, identification; C_t, cycle threshold; NA, not available.

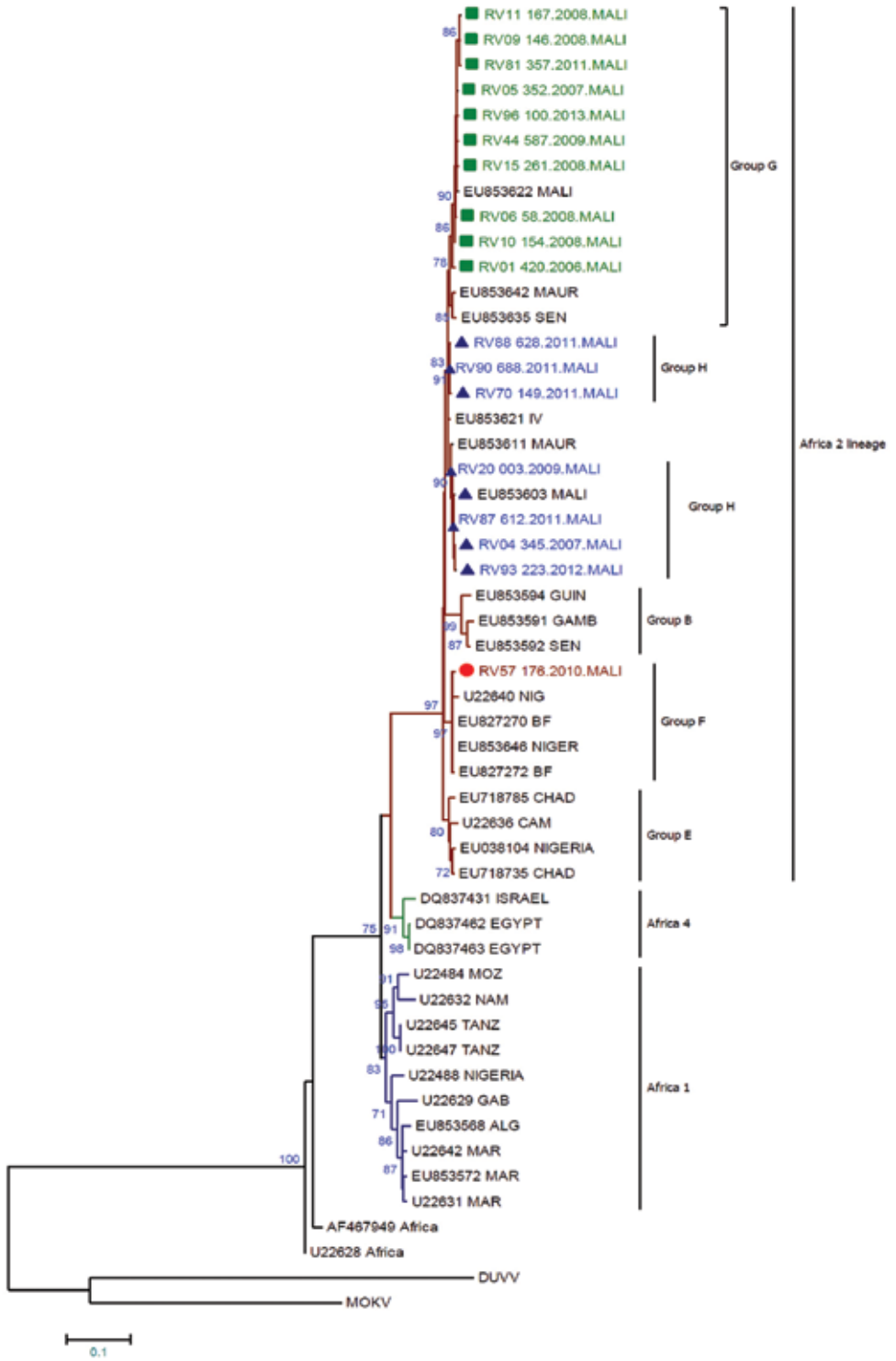

Figure 2. Maximum-likelihood phylogenetic tree based on a 564-nt sequence of nucleoprotein genes of 18 rabies virus sequences from Mali, 2002–2013, and representative sequences from Mali (n = 2), northern Africa (n = 6), South Africa (n = 2), West Africa (n = 32), and central Africa (n = 5). Sequences obtained in this study are identified in green, blue, and red. Green squares indicate genotype G, blue triangles indicate genotype H, and red circles indicate genotype F. The tree is rooted with 2 bat isolates used as outgroups Duvenhage virus (DUVV) (U22848) and Mokola virus (MOKV) (U22843). Bootstrap values (100 replicates) >70% are shown next to nodes. Alg, Algeria; BF, Burkina Faso; Cam, Cameroon; Gab, Gabon; Gamb, Gambia; Guin, Guinea; Maur, Mauritania; Mar, Morocco; Moz, Mozambique; Nig, Niger; Sen, Senegal; Tanz, Tanzania. Scale bar indicates nucleotide substitutions per site.

fluorescent antibody test (*5*) and stored at –20°C for further analyses. We selected 100 samples (95 with positive results and 5 with negative results) for further testing on the basis of their geographic origin.

Supernatants (100 µL) of suspensions (10% wt/vol) of dog brains were deposited on test paper cards, which stabilize nucleic acids. Virus RNA was extracted from stabilized samples by using the Iprep PureLink Virus Kit (Invitrogen, Paris, France) and subjected to partial nucleoprotein gene amplification of a conserved sequence (positions 55–660) (*9*). Virus RNA was tested by using a hemi-nested reverse transcription PCR (RT-PCR) and a real-time

Table 2. Characteristics of representative nucleoprotein gene sequences for rabies virus isolates, Mali, 2006–2013*

Isolate	Haplotype	Identical sequences (–546 nt of the N gene)	Phylogroup
RV09	2	RV50, RV56, RV51, RV19, RV79	G (Africa 2)
RV96	6	RV84, RV95	G (Africa 2)
RV90	11	RV67, RV60, RV68, RV88, RV89, RV22	H (Africa 2)
RV87	14	RV14, RV27	H (Africa 2)

*All identical sequences have 100% nucleoprotein (N) gene identity on the basis of 546 nt (positions 71–618) compared with the reference isolate. RV, rabies virus.

quantitative RT-PCR (*10*). After amplification, PCR products were sequenced in both directions by Beckman Coulter Genomics (Takeley, UK) and specific primers. A total of 32 stabilized samples showing positive results by heminested RT-PCR and real-time, quantitative RT-PCR were used for phylogenetic analyses (Table 1).

We constructed a maximum-likelihood phylogenetic tree (Figure 2) that excluded 15 duplicate sequences (Table 2) by using MEGA version 6 software (*11*). We also constructed a maximum-parsimony haplotype network by using TCS version 1.21 software (*12*).

We analyzed phylogenetic relationships between 18 partial nucleoprotein gene sequences and 31 representative sequences of Africa lineages of rabies virus. This analysis (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/22/5/15-0470-Techapp1.pdf) showed that all samples that belonged to the Africa 2 lineage were widely distributed in western and central Africa (*7*), including Mali and neighboring countries (Mauritania, Guinea, Senegal, Niger, Nigeria, Côte d'Ivoire, and Burkina Faso).

We found <2.1% divergence between all isolate sequences. For 17 haplotypes, 10 sequences were identified as belonging to subgroup G; this subgroup also included 3 sequences from Mali, Mauritania, and Senegal. Seven sequences (forming 6 haplotypes; RV88 was identical to RV90) belonged to subgroup H, which contained representative sequences from Côte d'Ivoire, Mauritania and Mali. One sequence from Mali (isolate RV57) belonged to subgroup F, which was similar to sequences from neighboring countries (Niger and Burkina Faso). Our data indicate that subgroup H might contain 2 distinct groups (online Technical Appendix Figure 2).

Analysis of the nucleoprotein gene identified canine rabies subgroups G and H in Mali, as reported (*7*), and subgroup F, which was found throughout Burkina Faso and Niger (*8*). Subgroup G circulates in Mauritania, Burkina Faso, and Senegal. Subgroup H contains viruses from Mauritania, Mali, Burkina Faso, and Côte d'Ivoire. The RV57 isolate included in subgroup F was isolated from a rabid dog at the border with Niger in 2010. Strong nucleotide identity (99.6%) was shown between RV57 and the strain isolated in Niger in 2010 (Genbank accession no. EU853646).

Conclusions

We identified 3 subgroups of the Africa 2 lineage of rabies virus in Mali. The presence of subgroup F could be explained by the movement of rabid animals across country borders. Previous studies reported rabies virus transmission by human-mediated animal movements (*13*,*14*). The information we obtained in this study should be useful for development of mass vaccination campaigns for dogs and eventual large-scale control programs in this country.

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Fatal Monocytic Ehrlichiosis in Woman, Mexico, 2013

Carolina G. Sosa-Gutierrez, Fortino Solorzano-Santos, David H. Walker, Javier Torres, Carlos A. Serrano, Guadalupe Gordillo-Perez

Human monocytic ehrlichiosis is a febrile illness caused by *Ehrlichia chaffeensis*, an intracellular bacterium transmitted by ticks. In Mexico, a case of *E. chaffeensis* infection in an immunocompetent 31-year-old woman without recognized tick bite was fatal. This diagnosis should be considered for patients with fever, leukopenia, thrombocytopenia, and elevated liver enzyme levels.

Ehrlichia are rickettsia-like intracellular bacteria of human medical and veterinary importance. The first cases of human monocytic ehrlichiosis (HME) were described in 1987, and the etiologic agent was subsequently identified in the United States as *Ehrlichia chaffeensis*, a strictly intracellular bacterium belonging to the family *Anaplasmataceae (1*). *E. chaffeensis* is transmitted by *Amblyomma americanum* ticks; reservoirs include domestic and wild animals (*2*,*3*). In the United States, most cases occur from April through September. Ehrlichiosis is usually self-limiting with nonspecific symptoms similar to those of influenza: fever, malaise, headache, and myalgia. Leukopenia is found in 60%–70% of patients, thrombocytopenia in 60%, and mild to moderate elevation of serum transaminase levels in 80%–90% (*4*,*5*). Serologic evidence of infection may be absent during the early acute phase of illness. In patients with illness severe enough that they seek medical attention, 50% require hospitalization and 2%–3% die (*6*).

In Mexico, only 1 case of *E. chaffeensis* has been reported (*7*). Recently, *E. chaffeensis* has been identified in *Rhipicephalus sanguineus* and *Amblyomma cajenennse* ticks, which are found throughout Mexico (*8*). We report another case in Mexico, this one fatal.

The Study

In late August 2013, a previously healthy 31-year-old woman from Estado de Mexico, in central Mexico, was admitted to an emergency department with a history of fever for 15 days, chills, muscle aches, malaise, loss of appetite, and headache. She had worked in a marketplace selling fruit and vegetables; she had not traveled abroad in the previous 3 months and was not aware of having been bitten by a tick. At the time of physical examination, she was confused and had mild respiratory distress, hepatosplenomegaly, tachycardia, and blood pressure within reference range. Blood collected at the time of admission showed leukopenia (0.90×10^9 cells/L), neutropenia (0.31×10^9 cells/L), lymphocytopenia (0.59×10^9 cells/L), thrombocytopenia (76×10^9 platelets/L), anemia (hemoglobin 8.2 g/dL), and elevated serum concentrations of aspartate transaminase (2,748 IU/L) and alanine transaminase (350 IU/L). A full evaluation for sepsis (blood cultures, morphologic evaluation, and culture of bone marrow aspirate) was performed. The bone marrow aspirate contained no significant abnormalities. Computed tomography indicated hepatosplenomegaly and a small pericardial effusion; ultrasonography indicated bilateral nephromegaly; and echocardiography indicated a small pericardial effusion and an ejection fraction of 59%.

After these procedures were completed, the patient was transferred to the intensive care unit (ICU); 1 day later, she was stable and discharged to a regular hospital ward, at which time blood and bone marrow culture results were negative. No morulae were detected in smears of peripheral blood and bone marrow. At that time, the patient's mental status included confusion, a psychotic episode, and symptoms of anxiety; a psychiatrist prescribed benzodiazepines. Prednisone therapy was added for suspected hemophagocytic syndrome. The patient's condition deteriorated; she experienced bleeding and hemodynamic instability and persistent fever. Four days after initial ICU discharge, she was transferred back to ICU, where she received antimicrobial drug therapy consisting of levofloxacin, amikacin, and meropenem and a transfusion of erythrocytes, plasma, and platelets. On her third day in the ICU, the patient still had pancytopenia and elevated concentrations of aspartate transaminase (674 IU/L) and alanine transaminase (105 IU/L). Blood, liver, and spleen samples were evaluated by PCR for *Mycobacterium* spp., *Rickettsia* spp., *Ehrlichia* spp., and *Anaplasma phagocytophilum*. *E. chaffensis* was found in the blood sample, and a morula-like structure was observed in a liver biopsy sample (Figure 1, panel C). Treatment with doxycycline (100 mg/12 h) was initiated; 2 days later her fever abated, but hypovolemic shock resulting from

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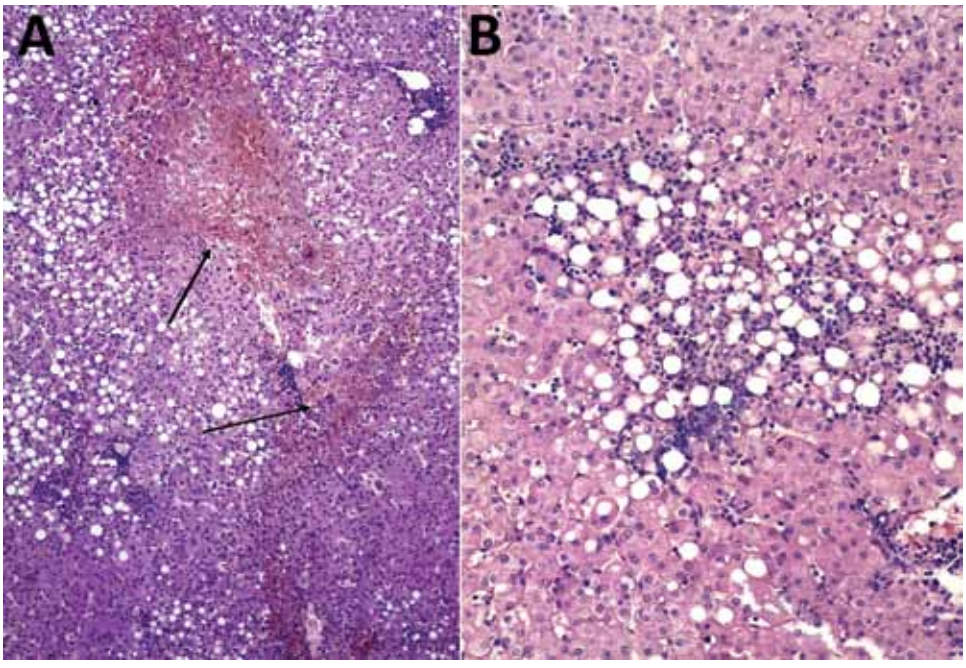

Figure 1. Histopathologic appearance of liver biopsy sample from woman with fatal human monocytic ehrlichiosis, Mexico, 2013. A) Necrotic hepatic lesions in a patchy distribution (arrows). Hematoxylin and eosin (H&E) stain; original magnification ×100. B) Macrovesicular steatosis and inflammatory lymphocytic infiltrate. H&E stain; original magnification ×200.

hemorrhage necessitated mechanical ventilatory assistance. On day 4 after initiation of doxycline, acute renal failure developed and hemodialysis was begun. On day 10, the patient experienced multisystemic failure with hemodynamic instability; despite inotropic support, she died.

Laboratory studies of a blood sample taken on the second day after the patient's original admission to hospital revealed no antibodies against hepatitis A, B, C, or E; parvovirus B-19; or HIV. PCR for *Mycobacterium* spp. was negative. Histopathologic study of the liver showed centrilobular hepatic necrosis, macrovesicular steatosis, and lymphohistiocytic inflammation (Figure 1).

After death, the diagnosis of HME was confirmed by nested PCR amplification of the 16S rRNA gene from spleen and liver tissues, by use of primers previously described (*8*). Sequencing of the PCR products showed 99.8% homology with *E. chaffeensis* str. Arkansas (GenBank accession no. KT308164). PCR results for *Anaplasma phagocytophilum* and *Rickettsia rickettsii* were negative.

Blood, serum, liver, and spleen samples were transferred to the Rickettsial and Ehrlichial Research Laboratory, University of Texas Medical Branch (Galveston, TX, USA), where 2 fragments of the *dsb* gene were amplified from blood, liver, and spleen DNA by real-time PCR as previously described (*9*). The amplicons showed 100% homology with *E. chaffeensis* str. Arkansas. ELISA and immunofluorescence assays (IFAs) were also performed. Serum antibodies were detected by ELISA (anti–tandem repeat proteins 120 and 32 of *E. chaffeensis*; titer 1:100) and by IFA (IgG; 1:512 titer) (Figure 2).

Conclusions

Góngora-Biachi et al. previously reported a probable case of HME in Mexico, diagnosed by IFA only (*7*). More recently, *E. chaffeensis* was detected in 5.5% of *Peromyscus* spp. rodents collected from 31 sites in Mexico (*8*). The presence of this pathogen in a wild host is evidence of a tick–vertebrate cycle and represents a potential risk for humans exposed to these tickborne rickettsiae.

The patient we report was hospitalized within 32 days of nonspecific clinical manifestations (leukopenia, anemia, thrombocytopenia, increased serum transaminase concentrations, and hepatosplenomegaly), which have been reported for persistent infection (*10*). Some authors have suggested that the clinical triad of leukopenia, thrombocytopenia, and elevated serum transaminase levels in a febrile patient without a rash is common for patients with HME (*11*). For this patient, multiorgan dysfunction and hematologic abnormalities persisted despite treatment with doxycycline. Death generally results from complications such as acute respiratory distress syndrome or sepsis with multiorgan failure (*12*).

Multiple neurologic manifestations have been reported for patients with ehrlichiosis, including severe headache, confusion, lethargy, hyperreflexia, clonus, photophobia, cranial nerve palsy, seizures, blurred vision, nuchal rigidity, and ataxia (*13*). The patient we report experienced changes in mental status, confusion, and a psychotic episode that was not improved by antimicrobial drug therapy.

To our knowledge, fatal cases of HME have not been reported in Mexico; they may have been ignored by clinicians or they may represent true emergence of the disease in Mexico. In a mouse model, a fatal course of infection has

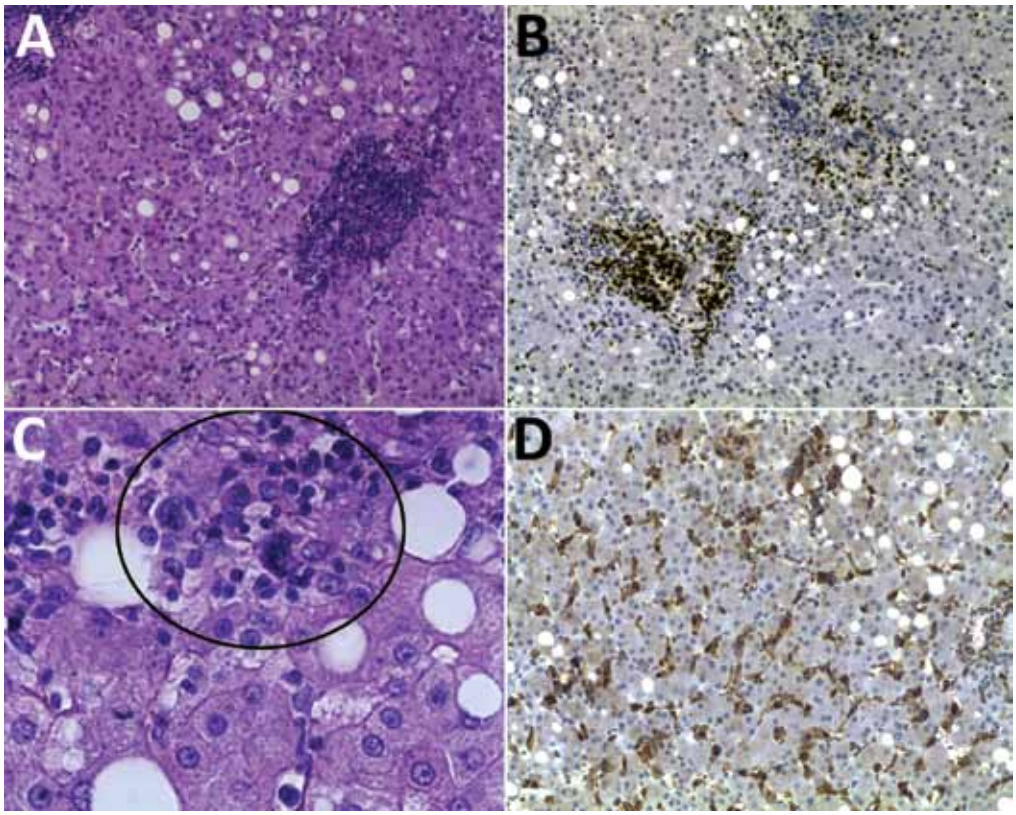

Figure 2. Histopathologic appearance of liver biopsy sample from woman with fatal human monocytic ehrlichiosis, Mexico, 2013. A) Clusters of cells in the liver lobule. Hematoxylin and eosin (H&E) stain; original magnification ×200. B) Immunohistochemical detection of T lymphocytes (CD3). Original magnification ×100. C) Multinucleated cells in parenchyma (circle). H&E stain; original magnification × 400. D) Immunohistochemical detection of macrophages and hyperplasia of Kupffer cells (CD68). Original magnification ×100.

been associated with an *Ehrlichia* strain that induces a toxic shock–like syndrome with high serum levels of tumor necrosis factor α (*14*). Fatal infection is often confounded because the signs and symptoms can mimic findings commonly associated with other infections, such as dengue fever, Rocky Mountain spotted fever, murine typhus, or other misdiagnosed febrile diseases (*15*) that are common in Mexico.

Illness caused by *Ehrlichia* spp. results in nonspecific signs and symptoms, and diagnosis requires a high index of suspicion (*8*). Seasonality should be taken into account; most new cases in humans occur during the summer, when tick activity is highest. Serologic evidence of infection may be absent during the early acute phase of illness (*15*), but use of PCR may help confirm suspected diagnoses. In Mexico, the possibility of *E. chaffeensis* infection should be investigated for patients with febrile illness, leukopenia, thrombocytopenia, and elevated liver enzymes; early diagnosis and timely treatment may prevent death.

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Ms. Sosa-Gutierrez is a PhD candidate from Universidad Nacional Autónoma de México. Her research focuses on eco-epidemiology of tickborne rickettsial pathogens as well as vector–pathogen–host interactions, distribution, and risk factors.

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Increased Rotavirus Prevalence in Diarrheal Outbreak Precipitated by Localized Flooding, Solomon Islands, 2014

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Flooding on 1 of the Solomon Islands precipitated a nationwide epidemic of diarrhea that spread to regions unaffected by flooding and caused >6,000 cases and 27 deaths. Rotavirus was identified in 38% of case-patients tested in the city with the most flooding. Outbreak potential related to weather reinforces the need for global rotavirus vaccination.

Pacific Island nations are vulnerable to extreme weather events that are projected to increase in severity and frequency with global climate change and can be associated with substantial health impacts, including outbreaks of diarrheal illness and other diseases (*1–4*). During the first week of April 2014, a tropical depression caused extensive flooding in the city of Honiara (population 64,609, 2009 census) and the surrounding province of Guadalcanal (population 158,222). Honira is the capital city of the island nation of the Solomon Islands (population 515,870), a nation which consists of 9 provinces and 992 islands in the Western Pacific (Figure 1, panel A). The flooding displaced >10,000 residents into emergency evacuation shelters and causing 22 deaths by drowning and other injuries (*5*). On April 20, 2014, an outbreak of diarrhea was declared in Honiara (Figure 1, panel B). During the next 2 months, diarrhea outbreaks and diarrhea-related deaths were reported from multiple provinces across the country that were not affected by the flooding. We investigated and report the flood-related outbreak of diarrhea in Honiara and its subsequent nationwide spread.

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The Study

During March 31–April 6, 2014, in Honiara, a tropical depression caused 663 mm of rainfall, 10 times the mean weekly rainfall recorded during 2010–2013 (*6*). After the flooding event, use of the Pacific Syndromic Surveillance System (PSSS) (*7*), which collects weekly aggregated data on diarrhea and other syndromes from 4 health facilities in Honiara and 4 provincial hospitals, was transitioned to a postdisaster Early Warning Alert and Response Surveillance Network (EWARN) to enhance outbreak detection and response (*8*).

To characterize the outbreak, we identified nonbloody (>2 loose bowel movements in 24 hours) and bloody (any episode of loose bowel movement with visible blood) diarrhea from PSSS/EWARN databases and patient registries of 10 outpatient and inpatient facilities in Honiara and 6 provincial hospitals from the outbreak period, April 7–July 13, 2014. To verify that this was an outbreak and not normal seasonal fluctuation, we also reviewed longitudinal diarrhea data from PSSS sentinel sites during January 2013–August 2015.

The National Referral Hospital (NRH) in Honiara evaluated fecal samples from inpatients and outpatients who had diarrheal illness. Samples were routinely tested for *Salmonella enterica*, *Shigella* spp, *Vibrio cholera*, and rotavirus. A rapid diagnostic test (RDT), the SD BIOLINE Rota/Adeno Rapid kit (Standard Diagnostics, Inc., Yongin, South Korea), was used to test for rotavirus at the NRH (*9*). We genotyped rotavirus-positive samples as previously described (*10*) and calculated incidence rates using the Solomon Islands' 2009 census. Negative binomial regressions and Fisher exact testing evaluated differences in rates and proportions testing positive.

We identified 4,087 diarrhea cases from the city of Honiara during the outbreak period, of which 3,664 cases (90%) were nonbloody. The mean number of weekly cases of diarrhea among PSSS sites in Honiara increased from 81.7 to 236.5 (rate ratio 2.90, 95% CI 2.13–3.96) from baseline to outbreak and baseline periods. (Table 1). The highest attack rate during the outbreak occurred in the <5 years age group (32%), which was >14× that observed for the ≥5 years age group (2%). During the outbreak, 6 of 9 provinces in the Solomon Islands reported diarrhea outbreaks, comprising the flood-affected province of Guadalcanal and 5 provinces that were not

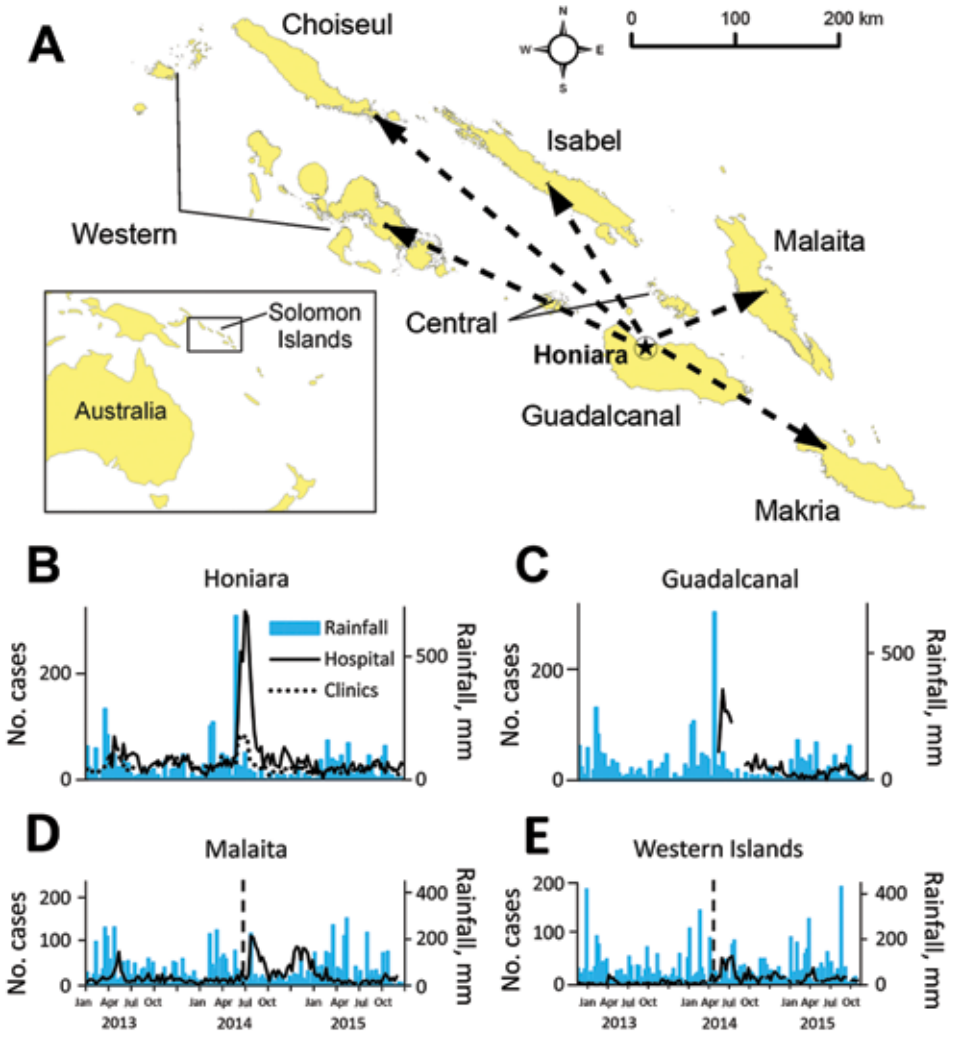

Figure 1. A) Spread of diarrheal disease in Solomon Islands after a postflooding outbreak in the capital city of Honiara, 2014, that resulted from a tropical depression. Dashed arrows indicate islands not affected by flooding where diarrheal outbreaks occurred. Two remote provinces, Temotu and Renell & Bellona, that did not report outbreaks are not included. B–E) Weekly rainfall measurements and outpatient diarrhea cases identified from the Pacific Syndromic Surveillance System (PSSS) database, December 31, 2012–August 30, 2015. The flooding occurred during April 3–5 in B) Honiara and C) other parts of Guadalcanal; vertical lines in panels D and E indicate the timing of the outbreak peak in Honiara and Guadalcanal. Cases of diarrhea are from PSSS weekly counts; Good Samaritan Hospital in Guadalcanal was designated as a PSSS site immediately postflood. Outpatient clinics in Honiara were Rove Clinic, Kukum Clinic, and Mataniko Clinic. Rainfall data presented for Guadalcanal Province is from Honiara.

affected by the tropical depression and flooding (Malaita, Makira, Western, Isabel, and Choiseul) (Figure 1, panels C–E). Hospital-based surveillance identified 4,407 diarrhea cases, of which 1,626 (37%) were reported from provinces unaffected by flooding. During the outbreak, 27 diarrhea-related deaths were identified, including 10 from flood-affected provinces (3.6 deaths/1,000 case-patients) and 17 from provinces where no flooding occurred (8.6 deaths/1,000 case-patients). Of the 23 deaths with information on age, 21 (91%) were <5 years.

Among 61 fecal samples collected during the outbreak in Honiara, 23 (38%) tested positive in the rotavirus RDT, versus none of the 43 samples collected during the same period in 2013 ($p<0.001$) (Table 2); the proportion positive for other pathogens tested was similar for 2013 versus 2014. Of 5 samples collected in June during the outbreaks in non-flood–affected areas, 4 tested positive in the rotavirus RDT; each of the 4 positive samples were obtained from a different province (Choiseul, Isabel, Makira, and Malaita). Isolates from the 4 samples were identified as genotype G9P[8] and found to have identical VP4 and VP7 sequences (GenBank Accession nos. KU312099–KU312102). Samples were not available for genotyping from flood-affected areas. The VP7 isolates were most similar to strains circulating in China and Russia during 2011–2013 (Figure 2).

Conclusions

We report a nationwide epidemic of diarrhea in the Solomon Islands, which was precipitated by a focal extreme weather event. Disasters can facilitate transmission of infectious diseases through population displacement, overcrowding, limited access to clean water, and compromised hygiene and sanitation (*3*). An assessment to quantify the risk for disease outbreaks was done immediately after the floods and identified a high risk for diarrheal outbreaks in Honiara and Guadalcanal (E.J. Nilles, unpub. data). Using protocols developed after a tsunami

Table 1. Comparison of diarrhea prevalence before, during, and after outbreak linked to localized flooding, Solomon Islands, 2013–2015*

Facility†	Facility type	Location‡	No. cases (mean weekly cases) 2013§	2014¶	2015#	Rate ratio (95% CI)
National Referral Hospital	Hospital	Honiara	470 (36.2)	1969 (140.6)	347 (24.8)	4.65 (3.24–6.75)
Kukum	Clinic	Honiara	290 (20.7)	675 (48.2)	256 (18.3)	2.47 (1.67–3.70)
Rove	Clinic	Honiara	288 (20.6)	487 (34.8)	264 (18.9)	1.76 (1.18–2.66)
Good Samaritan Hospital	Hospital	Guadalcanal	NA	812 (116.0)	253 (18.1)	6.41 (4.43–9.42)
Kilu'ufi	Hospital	Malaita	248 (17.7)	627 (44.8)	112 (8.6)	3.36 (2.17–5.29)
Taro	Hospital	Choiseul	4 (0.3)	48 (3.4)	31 (2.2)	2.74 (1.18–6.71)
Gizo	Hospital	Western	72 (5.1)	427 (30.5)	140 (10.8)	3.88 (2.37–6.52)

*Source: Pacific Syndromic Surveillance System (PSSS) database. The rate ratio was calculated by comparing rates during the outbreak in 2014 and rates during years without outbreaks (2013 and 2015) and using a negative binomial regression. NA, not available.
†One PSSS site in Honiara, Mataniko Clinic, is not shown because the clinic was partially destroyed by the flood and was not operational during the peak of the outbreak.
‡The city of Honiara is the capital of the Solomon Islands and is located in Guadalcanal Province; the remaining locations are provinces of the Solomon Islands.
§April 8–July 14, 2013 (14 weeks).
¶April 7–July 13, 2014 (14 weeks).
#April 6–July 12, 2015 (14 weeks).

emergency in 2013 (*11*) and insight gained by the experience, the Ministry of Health and Medical Services rapidly established an EWARN to strengthen disease detection and response capabilities. We report an increase in diarrheal cases shortly after the floods, and despite implementation of control measures, large outbreaks were detected in the flood-affected areas of Honiara and other parts of Guadalcanal. We subsequently identified outbreaks in multiple areas not affected by the flooding, indicating transmission from flood to non-flood affected areas.

Several factors suggest rotavirus was prevalent during the outbreak. The proportion of rotavirus-positive samples was 38% during 7 April–13 July, 2014, versus 0% during the same period in 2013, and increased to 55% during the peak of the outbreak from April 28–May 11. Outbreaks in non-flood affected provinces began soon after the peak of rotavirus transmission in Honiara. Illness and deaths caused by rotaviruses primarily affect the <5 years age group, unlike most other diarrheal agents with epidemic potential that affect all age groups (e.g., *Vibrio cholera, Shigella dysenteriae*, norovirus) (*12*). Finally, all 4 isolates from 4 non-affected provinces demonstrated 100% genetic homology of target genes, consistent with a common origin.

Table 2. Rotavirus rapid diagnostic tests of fecal samples before and during diarrheal outbreak precipitated by localized flooding, Honiara, Guadalcanal, Solomon Islands, 2013 and 2014*

Age group	% Positive (no. positive/no. tested) 2013†	2014‡	p value§
<5 y	0 (0/12)	41.9 (18/43)	0.005
≥5 y	0 (0/31)	23.5 (4/17)	0.012
Unknown	NA	100 (1/1)	NA
Total	0 (0/43)	37.7 (23/61)	<0.001

*Samples were collected and tested at the National Referral Hospital, Honiara; no rotavirus tests were conducted during the outbreak period in 2015. NA, not available.
†April 8–July 14, 2013 (14 weeks).
‡April 7–July 13, 2014 (14 weeks).
§By Fisher exact test.

However, the small number of diarrheal samples tested for a small number of pathogens limited our ability to conclusively define the role of rotavirus versus other diarrheal pathogens. There was limited historical baseline data against which to compare our findings, but given the consistent collection of data through the PSSS during 2013–15, and the direct observations of multiple study authors (who were involved in outbreak response activities) of substantial surges in diarrhea cases throughout the country, we are confident that this was an outbreak and not seasonal fluctuation. We only included ambulatory cases and did not evaluate hospitalizations.

Improved understanding of the health implications of changing climate patterns is necessary to drive evidence-based mitigation strategies. Post-disaster early warning alert and response networks can ensure the rapid detection of, and response to, disease outbreaks that are likely to increase as climate change leads to more severe extreme weather events. Countries at risk for extreme weather events and other disasters, including many Pacific island nations, should ensure protocols and plans have been tested and are in place and to rapidly enhance disease detection and response capacities. Rotavirus may cause or contribute to epidemics in the post-disaster setting, emphasizing the importance of implementing global childhood rotavirus immunization guidelines (*13*).

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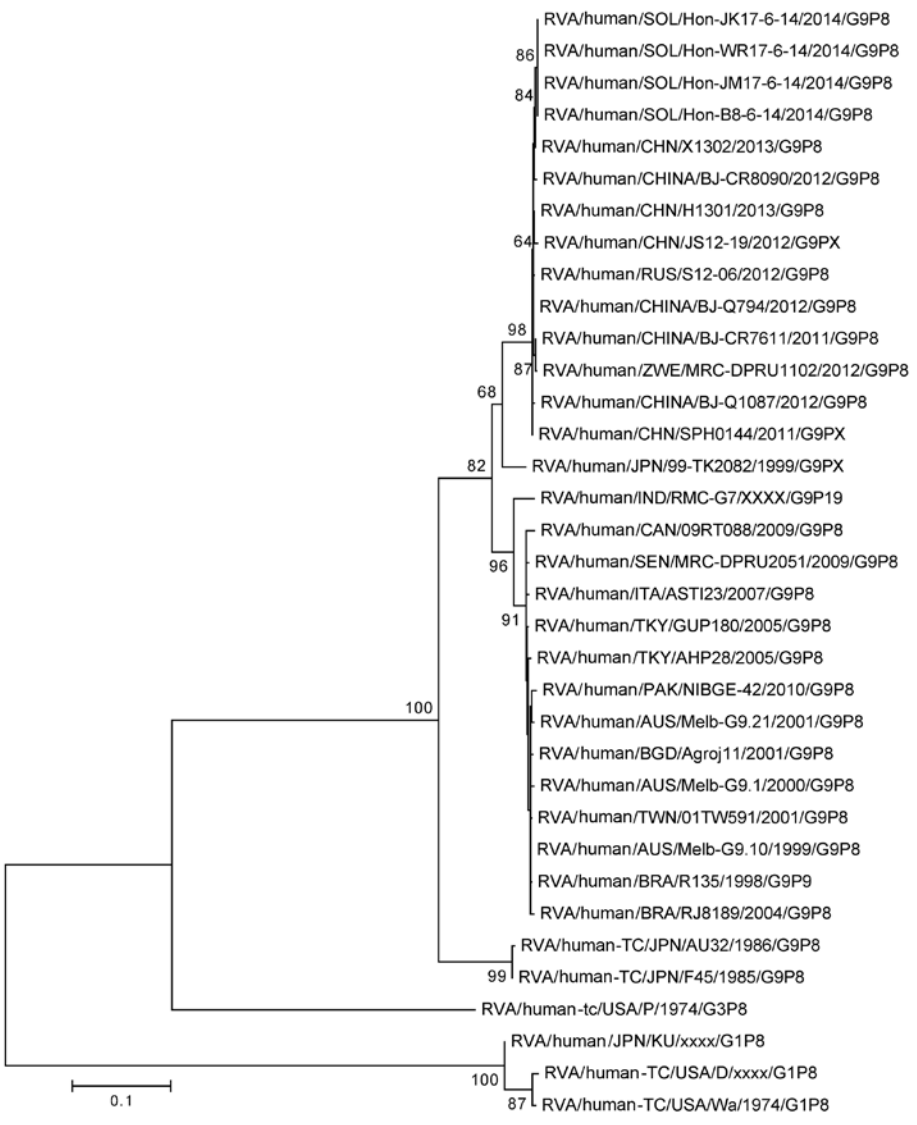

Figure 2. Nucleotide sequence–based phylogenetic tree of rotavirus viral protein (VP) 7 for isolates obtained in the Solomon Islands during an outbreak of diarrheal illness after flooding in the capital city of Honiara. Four isolates from different provinces had homologous VP7 sequences. We visually analyzed generated electropherograms and constructed contiguous DNA sequence files using the Sequencher Software program version 5.0.1 (Gene Codes Corp Inc., Ann Arbor, MI, USA). We performed nucleotide similarity searches using the BLAST (http://www.ncbi.nlm.nih.gov) and compared the nucleotide and deduced amino acid sequences of the VP7 gene with sequences available in GenBank possessing the entire open reading frame. We constructed multiple nucleotide and amino acid alignments using the MUSCLE algorithm in MEGA 6.0 (http://www.megasoftware.net/). Nucleotide and amino acid distance matrices were calculated by using the *p*-distance algorithm in MEGA 6.0. We selected the optimal evolutionary model based on the Akaike information criterion (corrected) ranking implemented in jModelTest (GitHub, Heidelberg, Germany) and generated maximum-likelihood phylogenetic trees using the nucleotide substitution model TrN+Gamma 4+I in MEGA 6.0, and assessed the robustness of branches by bootstrap analysis using 1,000 pseudoreplicate runs. Scale bar indicates base substitutions per site.

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Rickettsia sibirica mongolitimonae Infection, France, 2010–2014

Emmanouil Angelakis, Herve Richet, Didier Raoult

To further characterize human infections caused by *Rickettsia sibirica mongolitimonae*, we tested skin biopsy and swab samples and analyzed clinical, epidemiologic, and diagnostic characteristics of patients with a rickettsiosis. The most common (38%) indigenous species was *R. sibirica mongolitimonae*. Significantly more cases of *R. sibirica mongolitimonae* infection occurred during spring and summer.

Tickborne rickettsioses are zoonoses caused by spotted fever group (SFG) *Rickettsia* spp. (*1*). The first human infection with *R. sibirica mongolitimonae* was reported in France in 1996 (*2*). This patient had rope-like lymphangitis from the eschar to the draining lymph node, and *R. sibirica mongolitimonae* infection was thus named lymphangitis-associated rickettsiosis (*3*,*4*). Since then, other cases with or without rope-like lymphangitis have been described (*5*). Several SFG rickettsioses that have been considered nonpathogenic for decades are now associated with human infections, making these diseases useful as a paradigm for understanding emerging and reemerging infections (*6*). To further characterize human infections caused by *R. sibirica mongolitimonae*, we tested skin biopsy and swab samples and analyzed the clinical, epidemiologic, and diagnostic characteristics of patients with a rickettsiosis.

The Study

During 2010–2014, we tested skin biopsy (*7*) and cutaneous swab samples from rickettsiosis inpatients and outpatients throughout France. These samples were received frozen or in transport media; when possible, serum samples were also collected and sent at room temperature. For patients with positive *Rickettsia* results, epidemiologic and clinical data were collected.

We extracted total genomic DNA from samples by using a QIAamp tissue kit (QIAGEN, Hilden, Germany). We screened samples for *Rickettsia* spp. by using a quantitative PCR assay selective for a 109-bp fragment of a hypothetical protein (*8*). For positive samples, PCR amplification and sequencing selective for the *glt*A and *omp*A genes were performed (*8*). Samples were cultured in human embryonic lung fibroblasts (*9*). All serum samples were tested by immunofluorescence assay for SFG rickettsial antigens and typhus group rickettsiae (*10*). Student *t* or χ^2 tests were performed by using Epi Info version 6.0 software (Centers for Disease Control and Prevention, Atlanta, GA, USA). Means were compared by using analysis of variance or the Kruskal-Wallis test, on the basis of results of the Bartlett test for inequality of population variances. Proportions were compared by using the Mantel-Haenszel χ^2 or Fisher exact tests when the expected value of a cell was <0.05. *R. sibirica mongolitimonae* seasonality was assessed by using the autocorrelation module of PASW software version 17.02 (http://www.spss.com.hk/statistics/). $p < 0.05$ was considered significant.

We classified patients as definitively having a rickettsiosis if direct evidence of rickettsial infection was found on culture or molecular assays. Of 465 patients examined, 91 (20%) were infected with *Rickettsia* spp., most commonly *R. africae* (n = 36, 40%), followed by *R. conorii* (n = 21, 23%), *R. sibirica mongolitimonae* (n = 20, 22%), and *R. slovaca* (n = 14, 15%). Two cases of *R. sibirica mongolitimonae* infection in France have been reported (*11*,*12*).

For patients infected with *R. sibirica mongolitimonae*, median age ± SD (interquartile range) was 43 ± 21 (2–70) years, and most (12, 60%) were male (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/5/14-1989-Techapp1.pdf). The most common *Rickettsia* species in France was *R. sibirica mongolitimonae.* Only 1 patient mentioned recent travel to Spain; all others denied recent travel. Five patients mentioned recent outdoor activities, 8 mentioned frequent contact with dogs, and 1 mentioned contact with horses. A tick bite or tick handling was reported by 6 patients. An autocorrelation analysis revealed significant seasonality for *R. sibirica mongolitimonae* cases ($p<0.001$). Significantly more cases occurred during spring (April–June) (11 cases, 55%; $p = 0.006$), followed by summer (July–September) (8 cases, 40%, $p = 0.01$). One case occurred in October and none in winter.

The symptoms at disease onset included fever for all patients (duration 4–14 days), myalgia (n = 11, 55%), and headache (n = 3, 15%). Generalized maculopapular rash and an inoculation eschar developed in all patients. One patient had 3 eschars (buttocks, right hand, breast). A rope-like lymphangitis from the eschar to the draining lymph node was detected in 7 (35%) patients. One patient was admitted to an intensive care unit. For all 5 patients for whom an initial laboratory examination was available, increased liver enzymes (alanine aminotransferase, aspartate aminotransferase) and thrombocytopenia were found; 2 patients had hypoproteinemia. Oral doxycycline (7–14 days) was given to 19 patients; pristinamycin (7 days) was given to 1 patient. All outcomes were successful.

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Table. Epidemiologic and clinical characteristics of the main spotted fever group rickettsioses identified at the Unité de Recherche sur les Maladies Infectieuses et Tropicales Émergentes, Marseilles, France, 2010–2014*

Characteristic	*Rickettsia africae*	*R. conorii*	*R. slovaca*	*R. sibirica mongolitimonae*
No. cases	36	21	14	20
Geographic location	Zimbabwe and South Africa	Algeria, France, Morocco, Portugal, South Africa	France	France, Spain
Median age ± SD (IQR), y	58 ± 12 (31–80)	53 ± 18 (10–80)	36 ± 23 (6–65)	43 ± 21 (2–70)
Female sex	14 (39)	7 (33)	9 (64)	8 (40)
Recent travel	36	17	0	1
Clinical signs				
Fever	35 (97)	21 (100)	5 (36)	20 (100)
Rash	24 (67)	20 (95)	3 (21)	19 (95)
Enlarged lymph nodes	15 (42)	3 (14)	14 (100)	12 (60)
Lymphadenopathy location				
Cervical	1 (3)	3 (14)	14 (100)	5 (25)
Inguinal	14 (39)	0	0	3 (15)
Axillary	0	0	0	4 (20)
Eschar	36 (100)	18 (86)	14 (100)	20 (100)
Multiple eschars	13 (36)	0	0	3 (15)
Eschar location				
Scalp	0	2 (10)	14 (100)	0
Lower limbs	32 (89)	3 (14)	0	7 (33)
Upper limbs	2 (6)	2 (10)	0	4 (20)
Trunk	2 (6)	5 (24)	0	3 (15)
Neck	0	0	0	4 (20)
Lymphangitis	0	0	0	7 (35)
Treatment (duration, d)				
Doxycycline	34 (1–20)	21 (7–21)	12 (1–7)	19 (7–14)
Amoxicillin	2 (7)	None	None	None
Pristinamycin	None	1 (7)	None	1 (7)
Azithromycin	None	None	2 (4)	None

*Values are no. (%) patients unless otherwise indicated. IQR, interquartile range.

An eschar swab sample was available for 13 patients (*13*), and a skin biopsy sample was available for 10; all samples were positive for *R. sibirica mongolitimonae*. An acute-phase serum sample was also available for 13 patients; results of serologic testing were positive for only 2 (15%). A convalescent-phase serum sample was available from 5 patients; results were positive for 4 (80%). A skin biopsy sample was also positive for *R. sibirica mongolitimonae* by culture.

Statistical comparison of the 4 rickettsioses (Table) showed that a recent travel history was more common among patients with *R. africae* infection ($p<0.001$). *R. slovaca* infection was associated with absence of fever or rash ($p<0.001$ for each). Multiple eschars were associated with *R. africae* infection ($p<0.001$). An eschar on the neck was a characteristic of infection with *R. sibirica mongolitimonae* ($p = 0.002$); on the scalp, *R. slovaca* ($p<0.001$); on the trunk, *R. conorii* ($p = 0.05$); and on the lower limbs, *R. africae* ($p<0.001$). For patients with rope-like lymphangitis, the probability of *R. sibirica mongolitimonae* infection was 100% ($p<0.001$). Cervical lymphadenitis was associated with *R. slovaca* ($p<0.001$), inguinal lymphadenitis with *R. africae* ($p<0.001$), and axillary lymphadenitis with *R. sibirica mongolitimonae* infection ($p = 0.01$).

Conclusions

R. sibirica mongolitimonae is considered a rare pathogen; only 30 cases of infection with this organism have been reported in Europe and Africa (online Technical Appendix Table 2), of which 11 patients had lymphangitis, 27 inoculation eschars, and 18 a rash. In agreement with previous authors, we found that the most common signs of *R. sibirica mongolitimonae* infection were fever and rash. The addition of rope-like lymphangitis cases to those in the literature revealed that 17 (35%) of patients with *R. sibirica mongolitimonae* infection had this manifestation. Ramos et al. proposed that the term lymphangitis-associated rickettsiosis may be unwarranted for *R. sibirica mongolitimonae* infection because it is not found in all patients infected with this organism and because other rickettsioses produce lymphangitis (*14*). However, only *R. sibirica mongolitimonae* infection is associated with rope-like lymphangitis extending from the eschar to the draining lymph node; to our knowledge, only 1 case of mild, local, but not rope-like lymphangitis in a patient with *R. africae* infection has been described (*15*). In accordance with previous reports from France and Spain (*3*,*14*), we found that *R. sibirica mongolitimonae* infection was seasonal and that most cases occurred in the spring and summer.

Our strategy for diagnosing *Rickettsia* spp. infection on the basis of skin biopsy and cutaneous swab samples modified our knowledge of the epidemiology of SFG rickettsioses in France. We provide evidence that *R. sibirica mongolitimonae* infection is a frequent rickettsiosis, probably more frequent than *R. conorii* infection, which for decades has been considered the most common *Rickettsia* species in France.

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Q Fever, Scrub Typhus, and Rickettsial Diseases in Children, Kenya, 2011–2012

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To increase knowledge of undifferentiated fevers in Kenya, we tested paired serum samples from febrile children in western Kenya for antibodies against pathogens increasingly recognized to cause febrile illness in Africa. Of patients assessed, 8.9%, 22.4%, 1.1%, and 3.6% had enhanced seroreactivity to *Coxiella burnetii*, spotted fever group rickettsiae, typhus group rickettsiae, and scrub typhus group orientiae, respectively.

Rickettsial diseases, scrub typhus, and Q fever are increasingly recognized as key causes of undifferentiated fevers in humans in Africa (*1*–*6*). According to a recent study in western Kenya, respiratory viral infections were responsible for ≈41% of all fevers in children, but 37.1% were of unknown etiology. In the same study, malaria accounted for 5.2% of fevers (*7*). Several attempts have been made to elucidate causes of febrile illnesses in Kenya, but none have focused on causes among children, particularly in settings in which animal husbandry is integrated into communities. To further knowledge of the causes of febrile illnesses in western Kenya, we tested paired acute- and convalescent-phase serum samples from febrile children.

The Study

Participants were febrile, 1- to 12-year-old children brought for care at Webuye District Hospital (WDH; Bungoma, Kenya). Details of patient recruitment were previously reported (*7*). In brief, children with fever ≥37.5°C who lived within the administrative boundaries of Webuye Division were enrolled after informed consent was obtained. Acute-phase serum samples were stored at the time of enrollment. Patients returned ≈4 weeks after enrollment for follow-up physical examination and collection of convalescent-phase serum samples. The study protocol was approved by the Moi University Research and Ethics Committee (Eldoret, Kenya), WDH, and the Duke University Institutional Review Board (Durham, North Carolina, USA).

We used ELISAs as described (*5*,*8*–*10*) to evaluate the serum samples for IgG against spotted fever group rickettsiae (SFGR), typhus group rickettsiae (TGR), and scrub typhus group orientiae (STGO). In brief, all convalescent-phase and any unpaired acute-phase serum samples were screened (1:100 dilution), and screen-positive samples were titered in parallel with the corresponding acute-phase samples by serial dilution to assess enhanced seroreactivity to group-specific antigens. Enhanced seroreactivity was defined as a seroconversion from nonreactive in the acute phase to reactive (titer >400) in the convalescent phase or as a 4-fold rise in antibody titer between acute- and convalescent-phase serum samples. Results for samples that showed enhanced seroreactivity for STGO by ELISA were further confirmed by Western blot, using the recombinant proteins Kpr56 (*9*) and Kpr47b (*5*). The Kpr47b Western blot assay was performed as described (*5*) with the following modifications: Kpr47 antigen was loaded in a Mini-PROTEAN TGX Precast Gel and separated in a Mini Tetra cell electrophoresis module (both from Bio-Rad, Hercules, CA, USA). After being blocked for 1 hour, the membranes were incubated in serum samples diluted 1:100 in 10% blocking buffer and polyclonal *Escherichia coli* protein (ratio 1:1). Pierce ECL Plus Western Blotting Substrate (ThermoFisher Scientific, Waltham, MA, USA) was used to develop blots; signal was detected on a ChemiDoc XRS+ System (Bio-Rad).

To screen convalescent-phase serum samples, we used a *Coxiella burnetii* ImmunoDot assay (GenBio, San Diego, CA, USA) according to the manufacturer's instructions. Samples were diluted 1:200 in the initial stage of this immunoassay.

Data were imported into Stata 11.2 (StataCorp LP, College Station TX, USA) for analysis. We used χ^2 tests to compare categorical variables across groups, and we applied *t*-tests for continuous variables; $p \leq 0.05$ was considered significant.

A total of 370 febrile children were enrolled in the study during November 2011–December 2012. The average age was 4.4 (SD 2.8) years; 48.4% of enrollees were boys. The main symptom at first examination was fever (mean temperature 38.2°C [SD 0.6°C]). Most children had

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Table 1. Prevalence of IgG against *Coxiella burnetii*, SFGR, TGR, and STGO in serum samples from febrile children attending Webuye District Hospital, Bungoma, Kenya, November 2011–December 2012*

Antigen type	Total no. samples screened	No. (%) screen positive	Total no. paired samples	No. (%) IgG-positive†	No. (%) acute infections‡
C. burnetii	364	47 (12.9)	281	10 (3.6)	25 (8.9)
SFGR	364	104 (28.6)	281	23 (8.2)	63 (22.4)
TGR	364	6 (1.6)	281	1 (0.4)	3 (1.1)
STGO	364	21 (5.8)	281	9 (3.2)	10 (3.6)

*The children were 1–12 years of age. SFGR, spotted fever group rickettsiae; STGO, scrub typhus group orientiae; TGR, typhus group rickettsiae.
†No change in antibody titer between acute- and convalescent-phase serum samples.
‡Seroconversion or 4-fold rise in antibody titer.

been ill for 2 days before arrival at WDH, and 20% had been pretreated with an antimalarial drug, an antimicrobial drug, or both. Average time between enrollment and follow-up was 45 (SD 12) days. Overall, 364 convalescent- and unpaired acute-phase serum samples were screened; 47 (12.9%), 104 (28.6%), 6 (1.6%), and 21 (5.8%) were seropositive for *C. burnetii*, SFGR, TGR, and STGO, respectively. Of the 364 serum samples, 281 (77.2%) represented paired acute- and convalescent-phase samples, of which 25 (8.9%), 63 (22.4%), 3 (1.1%), and 10 (3.6%) had results indicative of acute *C. burnetii,* SFGR, TGR, and STGO infection, respectively (Table 1). Endpoints were 400 to ≥6,400 for SFGR, 400 to 1,600 for STGO, and 400 for TGR. Dual infections were noted in 27 (9.6%) of the 281 paired acute- and convalescent-phase samples: STGO and SFGR were found in 7 (2.5%), *C. burnetii* and SFGR in 14 (5.0%), *C. burnetii* and TGR in 2 (0.7%), and *C. burnetii* and STGO in 4 (1.4%) samples.

To ensure no cross reactivity of *C. burnetii*– and STGO-positive serum samples, we used Western blot to test the *C. burnetii*–positive serum against STGO proteins. No cross-reactivity was observed with the *C. burnetii*–specific IgG–positive serum and the *Orientia*-specific recombinant proteins (Figure).

Statistical analyses showed SFGR-infected children were more likely to be girls than boys (p = 0.007), indicating sex as a possible risk factor (Table 2). The primary clinical signs of all infections were cough, diarrhea, vomiting, and inability to drink or breastfeed. *C. burnetii*–infected children were more likely than *C. burnetii*–uninfected children to report cough (p = 0.018). Of the children with rickettsial infections, 81%–90% received antimalarial drugs and ≈60% received antimicrobial drugs; similar proportions were seen for Q fever patients. Virtually all Q fever and rickettsia-infected patients received a diagnosis of malaria by the treating clinician. *C. burnetii* and SFGR cases were detected throughout the year, with peaks noted in February–June for SFGR and April–July for *C. burnetii*.

Conclusions

In this study, the seroprevalence of IgG against SFGR was 28.7%, a finding similar to that in a previous study in Tanzania (*11*). We observed higher rates (22.4%) of acute SFGR infections than were observed among pediatric patients in Tanzania (≥8%), where similar serologic tools were used to diagnose acute rickettsial infections (*11*,*12*). Acute SFGR infections occurred throughout the year, but increases were noted during February–June, months which coincide with the long rainy season in Kenya. Seasonal peaks of SFGR infections coinciding with wetter months have been observed in sub-Saharan

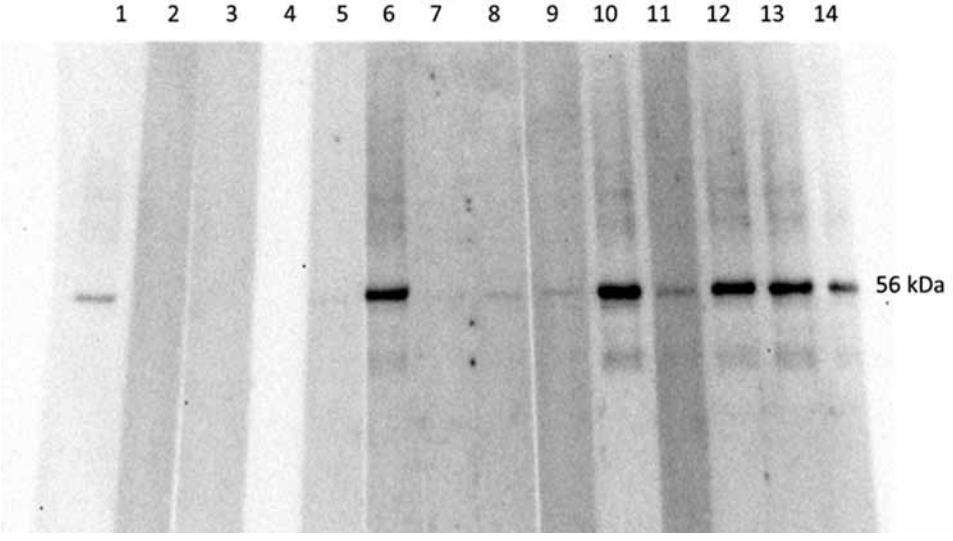

Figure. Western blot analysis, using *Orientia* 56Kpr recombinant protein, of serum samples from febrile children in western Kenya, November 2011–December 2012. Lane 1, positive control; lane 2, negative control; lanes 3–4, *Coxiella burnetii*–positive patients; lane 5, *Orientia* spp.–negative patient; lanes 6–14, *Orientia* spp.–positive patients.

Table 2. Analysis of variables across *Coxiella burnetii*, SFGR, and STGO infections in febrile children attending Webuye District Hospital, Bungoma, Kenya, November 2011–December 2012*

Variable	*C. burnetii*			SFGR			STGO		
	Positive, n = 25†	Negative, n = 256†	p value	Positive, n = 63†	Negative, n = 218†	p value	Positive, n = 10†	Negative, n = 271†	p value
Mean age, y (SD)	3.8 (0.48)	4.5 (0.18)	0.283	3.8 (0.35)	4.6 (0.19)	0.065	4.8 (0.87)	4.4 (0.17)	0.669
Sex									
M	39	47	0.452	31.8	51.1	0.007‡	20	47.6	0.086
F	61	52		68.2	48.9		80	52.4	
Symptom									
Rash	0	1.6	0.548	1.6	1.4	0.901	0	1.5	0.7
Vomiting	21.7	16.7	0.536	25.4	14.7	0.046§	30	16.6	0.269
Cough	73.9	48	0.018#	54	49.1	0.495	60	49.8	0.527
Diarrhea	17.4	10.5	0.31	15.9	9.6	0.164	30	10.3	0.051
Difficulty breathing	0	1.6	0.548	1.6	1.4	0.901	0	1.5	0.7
Inability to drink or breastfeed	13	7.4	0.331	9.5	7.3	0.57	10	7.8	0.795
Admitted to hospital	30.4	23.6	0.466	20.6	25.2	0.453	10	24.7	0.286
Treatment administered at hospital									
Antimicrobial drug	73.9	54.7	0.074	61.9	59.7	0.746	50	57.2	0.505
Antimalarial drug	82.6	81	0.851	81	81.2	0.966	90	80.8	0.466
Recovered after treatment	100	92.6	0.158	98.4	92.3	0.076	100	92.7	0.372
Malaria diagnosed by clinician	100	89.8	0.094	95.2	89.1	0.138	100	90.2	0.294

*SFGR, spotted fever group rickettsiae, STGO, scrub typhus group orientiae.
†Values are percentages unless otherwise specified.
‡Being a girl was indicated as a possible risk factor for SFGR infection ($p<0.05$).
§Children with SFGR were more likely to report vomiting ($p<0.05$).
#Children with *C. burnetii* were more likely to report cough ($p<0.05$).

Africa (*3*). A higher frequency of acute SFGR was noted in girls than boys and may be related to occupational exposure, as noted among women in Peru (*13*). TGR rates in our study are comparable to those reported in Tanzania (*11*).

The seroprevalence of STGO was 5.8%, similar to that reported from a previous study in Kenya (*5*). Detection of enhanced seroreactivity to scrub typhus suggests acute infections with *Orientia* spp. Scrub typhus was formerly thought to be geographically restricted to Asia, but the distribution has been redefined by the recent detection of *Orientia* DNA in mice in Africa; description of scrub typhus–like illnesses in Chile and United Arab Emirates; and discovery of a new species, *O. chuto,* isolated from a patient who had visited Dubai (*14*).

In addition, the prevalence of antibodies to *C. burnetii* observed in our study is higher (12.9%) than that reported for other African countries (<8%) but within the range reported among children in Egypt (10%–32%) (*15*). Our findings showed a much higher rate (8.9%) of acute Q fever than reported among infants and children in Tanzania (2.6%) (*11*). A distinct seasonality was noted with acute Q fever infections, and this may be related to the parturient season in domestic and wild animals (*11*). The dual infections with SFGR and STGO and STGO and *C. burnetii* may be the result of similar risk factors. Additional research is needed to identify the reservoirs and vectors of *Orientia* spp. in Africa and to identify key risk factors for infection.

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Dr. Maina is a postdoctoral research associate at the Naval Medical Research Center. Her research effort is focused on the epidemiological surveillance of emerging infectious diseases in multihost(s) systems in an effort to identify the arthropod vectors, reservoir hosts, and risk factors associated with these diseases.

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Molecular Characterization of Chikungunya Virus, Philippines, 2011–2013

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During 2011–2013, a nationwide outbreak of chikungunya virus infection occurred in the Philippines. The Asian genotype was identified as the predominant genotype; sporadic cases of the East/Central/South African genotype were detected in Mindanao. Further monitoring is needed to define the transmission pattern of this virus in the Philippines.

Chikungunya fever is a mosquitoborne infection that causes large outbreaks mainly in tropical and subtropical countries. The causative agent is chikungunya virus (CHIKV), an enveloped, single-stranded positive-sense RNA virus (family *Togaviridae*, genus *Alphavirus*). Phylogenetic analysis of the E1 gene of CHIKV revealed 3 genotypes: West African, East/Central/South African (ECSA), and Asian (*1*). In 2005, large outbreaks occurred in the islands in the Indian Ocean and India that were caused by the Indian Ocean lineage (IOL) virus, which newly emerged from the ECSA genotype (*2*). More recently, the emergence and potential spread of ECSA and Asian genotypes in the Americas have been major public health concerns in the region. (*3*).

In the Philippines, CHIKV was first isolated in 1965 (*4*). Since then, sporadic cases have been reported, including those among US Peace Corps volunteers stationed in the islands of Mindanao, Cebu, and Masbate in 1986 (*5*), and a local community outbreak in Cavite, Luzon Island, was reported in 1996 (*6*). However, the first nationwide CHIKV outbreak was identified starting in 2011 (Philippines Department of Health, unpub. data). Previous studies of chikungunya fever in the Philippines focused on clinical and serologic analyses (*7*), and recent reports on the molecular surveillance of CHIKV in the country were limited and analyzed only samples collected in 2012 (*8*,*9*). We conducted genetic analysis to characterize recent CHIKV infections that caused a large nationwide outbreak in the Philippines during 2011–2013.

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The Study

Serum samples were collected through the chikungunya fever surveillance under the Philippine Integrated Disease Surveillance and Response of the Department of Health Epidemiology Bureau from different provinces in the Philippines during 2011–2013. Samples were collected from patients suspected to have chikungunya fever manifesting with symptoms such as fever, rash, and arthralgia. Samples were sent to the Research Institute for Tropical Medicine, which serves as the National Reference Laboratory for Dengue and Other Arboviruses. We screened serum samples by using CHIKV IgM-capture ELISA (NovaLisa, NovaTec Immundiagnostica GmbH, Dietzenbach, Germany). We extracted viral RNA using the QIAamp Viral RNA kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and amplified the partial E1 gene using 1-step reverse transcription PCR followed by direct Sanger sequencing with primers as previously described (*10*,*11*). We conducted phylogenetic analysis using the maximum-likelihood method as implemented in MEGA 6 software (http://www.megasoftware.net/). Molecular clock analysis and Bayes factor calculation were performed using BEAST software 1.8.0 (http://beast.bio.ed.ac.uk/) to select the best migration event model of CHIKV among countries. Bayes factor analysis was used to test phylogeographic hypothesis whether posterior migration rate between locations in whole evolutionary history was significantly higher than the expected prior migration rate, assuming truncated Poisson probability (*12*).

A total of 5,729 serum samples were collected from persons suspected to have chikungunya within 5 days after symptom onset. Fever, rash, and arthralgia were the most common symptoms (53%, 47%, and 34% of patients, respectively). Of the 5,729 serum samples, 2,891 were IgM positive by ELISA. We conducted reverse transcription PCR on 382 representative samples among IgM-negative patients in accordance with the CHIKV outbreak surveillance strategy of the Philippines, of which 131 samples tested positive. Partial E1 gene sequence (733 nt) was obtained from 31 samples. Sequences were submitted to GenBank (accession nos. LC064714–LC064744).

Phylogenetic analysis identified 28 Asian genotype viruses and 3 ECSA genotype viruses (Figure 1). Sequence

[1]These authors contributed equally to this article.

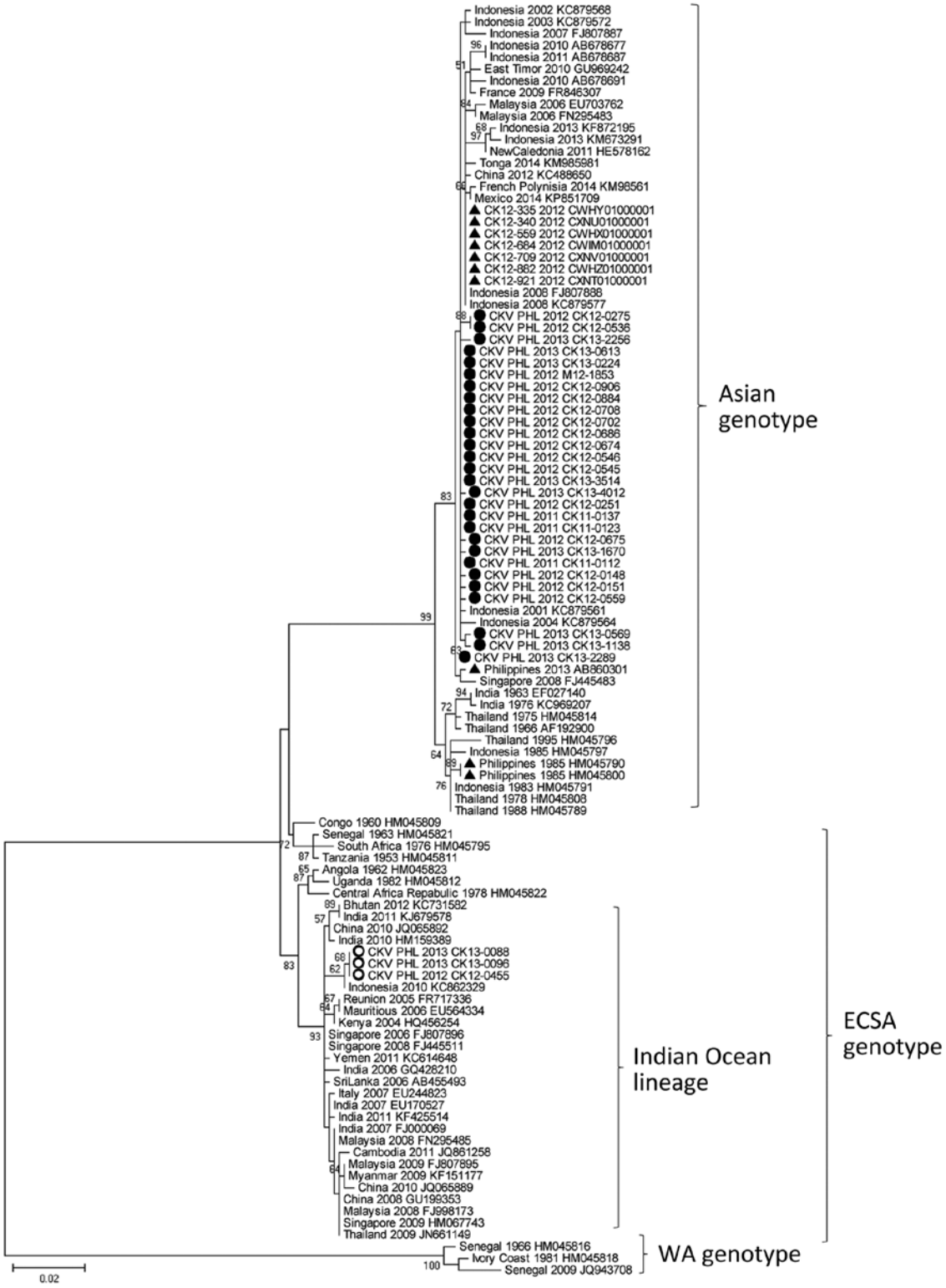

Figure 1. Phylogenetic analysis of partial (733 nt) E1 gene of 31 CHIKVs detected in the Philippines in this study during 2011–2013 compared with 77 global strains. The tree was constructed using maximum-likelihood method with the Kimura 2-parameter model and 1,000 bootstrap replications. Bootstrap values >50% are indicated on the branches of the tree. Black circles indicate Asian genotypes; open circles indicate ECSA genotypes analyzed in this study; triangles indicate reference strains collected in the Philippines. CHIKV, chikungunya virus; ECSA, East/Central/South African, WA, West African. Scale bar indicates nucleotide substitutions per site.

Table. Bayes factor of migration events of Asian genotype of chikungunya virus based on 65 cases, Philippines, 2011–2013*

Bayes factor	Locations
68.8	Philippines, Indonesia
68.0	India, Thailand
54.2	Indonesia, New Caledonia
25.6	French Polynesia, Mexico
22.0	France, Indonesia
19.5	Indonesia, Malaysia
11.3	East Timor, Indonesia
7.6	Indonesia, Singapore
6.8	Indonesia, Thailand
3.4	China, Tonga
3.3	China, Indonesia
2.3	East Timor, France
2.3	Philippines, Thailand
2.2	Mexico, Tonga
2.1	French Polynesia, Tonga
2.1	China, French Polynesia
2.0	Philippines, Tonga
1.9	China, Mexico
1.7	China, Philippines
1.3	Malaysia, Singapore

*The migrations of Asian genotype viruses between countries were determined by using Bayes factor analysis. Bayes factor value >5 is considered a significant migration.

analysis of E1 gene showed >99% nt similarity among 28 Asian genotype viruses (data not shown). Bayes factor analysis for the migration events also showed that migration might have occurred between Indonesia and Philippines with high probability (Table). CHIKV-positive patients were found only in Mindanao Island in 2011 but were detected in other parts of the country in 2012 and 2013. All CHIKV detected in parts of the country other than Mindanao were identified as Asian genotype.

The 3 ECSA genotype viruses were detected in the Davao Del Sur and Davao Oriental in Mindanao Island in 2012 and 2013, and all were clustered into IOL (Figures 1, 2). This finding indicates that the 2 genotypes co-circulated in the island during the outbreak. ECSA genotype viruses in the Philippines were closely related to the Indonesian viruses (GenBank accession no. KC862329) detected in 2010. Sequencing analysis showed that all Philippine ECSA viruses possess the alanine to valine substitution (A226V) in the E1 gene (online Technical Appendix Tables 1, 2, http://wwwnc.cdc.gov/EID/article/22/5/15-1268-Techapp1.pdf).

Conclusions

The recent large CHIKV outbreaks in other Asian countries were caused mainly by the IOL of ECSA genotype (*2*). However, the 2011–2013 outbreak in the Philippines was caused mainly by the Asian genotype; the reason that this large outbreak was caused by this genotype is unknown. Previous reports have confirmed the reemergence of Asian genotype viruses in the Philippines (*8,9*). In this study, phylogenetic analysis and Bayes factor calculation showed that the Philippines viruses were closely related to Indonesian viruses, which might explain why the outbreak started in southern Mindanao, near Indonesia. Although sequence data of CHIKV in the database are not enough to identify the exact origin of the virus, we tried to estimate when CHIKV was introduced into the Philippines with molecular clock analysis using the dataset of Asian genotype viruses (online Technical Appendix Figures 1, 2). The results suggested that circulating Asian genotype viruses in the Philippines were introduced from Indonesia before 2010.

We also detected IOL of ECSA genotype, which possesses the E1-A226V substitution, and its co-circulation with the Asian genotype in the Philippines. However, the geographic distribution of ECSA genotype in 2012 and 2013 was limited to southern provinces in Mindanao. After the large outbreak in the Indian Ocean region in 2005, IOL of ECSA genotype rapidly spread to Asian countries and then co-circulated with the endemic Asian viruses, then eventually became the predominant genotype (*13*). However, this circulation pattern differs from what we observed in the Philippines. Until the early 2010s, most of the viruses

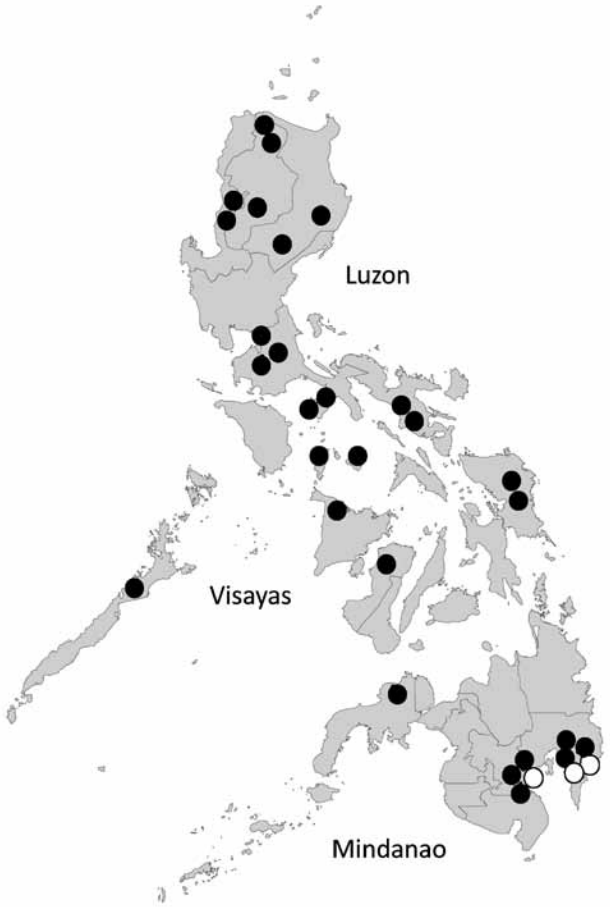

Figure 2. Geographic distribution of CHIKV genotypes in the Philippines. The location of samples collected in this study are indicated by circles; 1 circle represents 1 sample. Black circles indicate Asian genotype; white (open) circles indicate East/Central/South African genotype. CHIKV, chikungunya virus.

circulating in the Philippines and Indonesia were still Asian genotype, and ECSA genotype viruses had been reported in West Kalimantan, Indonesia, in 2011 (*14*) and in the Philippines in 2012 several years after the large outbreak in the Indian Ocean region (*2*). The movement of persons near the border of these countries might play a key role in CHIKV transmission.

As part of the national vector control program, surveillance of *Aedes aegypti* and *Ae. albopictus* mosquitoes has been conducted in several areas in the Philippines. A previous report showed that the proportion of these 2 mosquito species was almost the same in Metro Manila (*15*). If the proportion of *Ae. albopictus* mosquitoes increases, the ESCA genotype virus with A226V mutation could spread more rapidly in the country. Thus, monitoring the spread of ECSA genotype viruses and the proportion of the *Aedes* mosquitoes in the Philippines is important.

We have demonstrated that the Asian genotype CHIKV, which is closely related to the Indonesian viruses, was identified in Mindanao in 2011 and spread to other regions in 2012 and 2013. Like the Asian genotype, ECSA genotype virus was first detected in Mindanao in 2012. Mindanao might play a key role for the introduction of the CHIKV into the Philippines. Further monitoring is necessary to define the transmission pattern of CHIKV, including cross-border transmission.

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Severe Sepsis and Septic Shock Associated with Chikungunya Virus Infection, Guadeloupe, 2014

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During a 2014 outbreak, 450 patients with confirmed chikungunya virus infection were admitted to the University Hospital of Pointe-à-Pitre, Guadeloupe. Of these, 110 were nonpregnant adults; 42 had severe disease, and of those, 25 had severe sepsis or septic shock and 12 died. Severe sepsis may be a rare complication of chikungunya virus infection.

In November 2013, the first autochthonous cases of chikungunya virus (CHIKV) infection were identified in the territory of Saint-Martin in the French West Indies (1). Since that time, local transmission of the virus has been identified in nearly all Caribbean islands and in Central and South America (2). In Guadeloupe, an outbreak started in the first weeks of 2014 and ended by November 2014. No new definite case of chikungunya has been reported since January 2015. During the 2014 outbreak, ≈40% of the population (≈160,000 persons) became infected with CHIKV. However, the hospitalization rate for chikungunya was <0.5%. A total of 450 patients with CHIKV infection and a positive reverse transcription PCR (RT-PCR) test result for CHIKV were admitted to the University Hospital of Pointe-à-Pitre (UHPAP), Guadeloupe, and were hospitalized >24 hours. Of these 450 patients, 241 were children, 99 were pregnant women, and 110 were nonpregnant adults. The objectives of our study were 1) to describe the characteristics of nonpregnant adult patients who had atypical or severe forms of the disease and 2) to search for predictive factors for severe forms.

The Study

During the outbreak, a standardized case report form was filled out for each patient admitted to UHPAP who had clinical symptoms consistent with chikungunya and a positive CHIKV RT-PCR test result on a blood sample. The information recorded included demographics, preexisting comorbidities, which were summarized by using the Charlson index (*3*) and McCabe classification (*4*), and clinical manifestations described by organ system (e.g., cardio-circulatory, cerebral, respiratory, renal, and hepatic). Standard definitions were used for organ system failures and severe sepsis and septic shock (*5*).

The following laboratory parameters were retrieved from the patient's record in the hospital's electronic medical system: whole and differential leukocyte and platelet counts; corpuscular hemoglobin; and C-reactive protein, serum creatinine, alanine aminotransferase, aspartate aminotransferase, creatine kinase, and lactate dehydrogenase levels. For each parameter, 2 values were considered, the first value recorded within the first 24 hours of admission and the most abnormal value observed from hospital day 2 through day 7. After hospital discharge or death, each case of CHIKV infection was categorized as one of the following: 1) a common form, in which only fever or arthralgia occurred; 2) an atypical form, in which ≥1 organ system was involved; and 3) a severe form, in which the patient had ≥1 organ system failure or had been admitted to the intensive care unit.

Of the 110 nonpregnant adults hospitalized with chikungunya who had a positive CHIKV RT-PCR test result, 34 had a common form, 34 had an atypical form, and 42 had a severe form. Overall, the characteristics of patients with common and atypical forms were similar. Therefore, we compared the characteristics of the 48 patients with severe forms with those of the 68 patients with nonsevere forms (i.e., common and atypical forms) by selected demographic, clinical, laboratory, and outcome characteristics. Patients with severe forms were not older and did not have more comorbidities than patients with nonsevere forms. At hospital admission, the rates of classical signs of chikungunya, such as fever and peripheral arthralgia, were similar in both groups. Patients with severe forms had significantly less occurrence of headache but increased occurrence of acute cardiac failure; they also had occurrence of organ dysfunction significantly more often than did patients with nonsevere forms (Table 1). As for laboratory abnormalities, patients with severe forms had significantly higher whole leukocyte counts, polymorphonuclear cell counts, and serum lactate dehydrogenase levels at baseline and within the first week after admission (Table 1).

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Table 1. Selected characteristics of 110 nonpregnant adult patients with chikungunya virus infection, by severity of disease, University Hospital of Pointe-à-Pitre, Guadeloupe, French West Indies, January–November 2014*

Characteristic	Nonsevere, n = 68	Severe, n = 42	p value†
Baseline characteristics			
Median age, y (interquartile range)	71 (59–80)	68 (58–77)	0.27
Male sex	36 (53)	26 (62)	0.36
Preexisting comorbid conditions			
Immune suppression	4 (6)	1 (2)	0.69
Diabetes mellitus	28 (41)	16 (38)	0.75
Chronic heart disease	9 (14)	11 (28)	0.07
Cerebrovascular disease	6 (9)	1 (3)	0.35
Chronic lung disease	1 (2)	2 (5)	0.66
Chronic liver disease	0	1 (3)	0.8
Chronic renal disease	5 (8)	4 (10)	0.95
Cancer	5 (8)	2 (5)	0.92
Charlson index, median (interquartile range)	4 (3–5)	4 (1–5)	0,41
McCabe class 1	50 (77)	24 (62)	0.09
Clinical symptoms, present on admission to hospital			
Arthralgia/arthritis	49 (82)	30 (83)	0.84
Headache	21 (39)	3 (9)	0,002
Fever	61 (92)	35 (83)	0.25
Myalgia	24 (48)	13 (41)	0.41
Cardiac manifestations	10 (15)	28 (67)	<0.001
Central nervous system manifestations	20 (30)	13 (31)	<0.001
Respiratory manifestations	9 (14)	29 (71)	<0.001
Hepatic manifestations	1 (2)	12 (29)	<0.001
Renal manifestations	12 (19)	20 (50)	0.001
Organ failures, at any time of the course of the disease			
Cardio-circulatory failure	0	22 (52)	<0.001
Neurologic failure	0	6 (14)	0.006
Respiratory failure	0	21 (50)	<0.001
Liver failure	0	9 (21)	<0.001
Renal failure	0	15 (36)	<0.001
Laboratory data, median (interquartile range)			
Whole leukocytes, day 1, G/L	5.80 (4.00–7.00)	7.50 (4.90–13.00)	0.01
Whole leukocytes, days 2–7, G/L	3.00 (2.10–5.40)	8.70 (3.20–14.70)	<0.001
Polymorphonuclear neutrophils, day 1, G/L	4.16 (2.82–5.43)	5.59 (3.40–10.94)	0.01
Polymorphonuclear neutrophils, days 2–7, G/L	1.63 (1.05–5.02)	7.13 (2.32–11.82)	<0.001
Hemoglobin, day 1, g/dL	12.6 (11.3–13.6)	12.1 (10.2–13.5)	0.25
Hemoglobin, days 2–7, g/dL	11.9 (11.3–13.6)	8.6 (10.5–11.6)	<0.001
Platelets, day 1, G/L	178(125–233)	147 (107–199)	0.13
Platelets, days 2–7, G/L	160 (110–200)	108 (75–189)	0.04
C-reactive protein, day 1, mg/L	36 (20–79)	43 (16–75)	0.57
C-reactive protein, days 2–7, mg/L	70 (33–106)	94 (37–167)	0.24
Lactate dehydrogenase, day 1, IU/L	286 (207–354)	579 (310–1135)	0.001
Lactate dehydrogenase, days 2–7, IU/L	363 (248–418)	422 (335–1600)	0.04
Creatine kinase, day 1, IU/L	268 (132–808)	395 (237–740)	0.36
Creatine kinase, day 2, IU/L	973 (163–2826)	683 (240–2164)	0.85
Aspartate aminotransferase, day 1, IU/L	34 (25–48)	50 (30–127)	0.01
Aspartate aminotransferase, days 2–7, IU/L	28 (19–58)	64 (33–351)	0.2
Alanine aminotransferase, day 1, IU/L	20 (15–30)	31 (21–57)	0.02
Alanine aminotransferase, days 2–7, IU/L	28 (19–58)	40 (20–234)	0.1
Creatinine, day 1, µmol/L	95 (78–131)	119 (81–182)	0.07
Creatinine, days 2–7, µmol/L	101 (81–139)	153 (75–410)	0.09
Outcome			
Death	1 (2)	13 (31)	<0.001

*Values are no. (%) except as indicated. All patients were hospitalized and had infection confirmed by reverse transcription PCR.
†p value denotes the comparisons between the 42 patients with severe forms and the 68 patients with nonsevere forms, based on nonparametrical tests.

Among the 42 patients who had a severe form of the disease, 25 patients had illness consistent with the case definition for severe sepsis and had no other identified cause for this syndrome but CHIKV, according to blood and urine cultures, which had been performed systematically in all these patients. Overall, the background characteristics of these 25 patients were not significantly different from those of the other 85 patients (Tables 1, 2). At admission to hospital, these 25 patients had significantly higher occurrence of cardiac, respiratory, and renal manifestations and had significantly higher leukocyte counts and levels of serum lactate dehydrogenase, aspartate aminotransferase, and creatinine, which are clinical and laboratory indicators of sepsis, than did patients without severe sepsis or septic shock (Table 2). In addition, their

Table 2. Selected characteristics of 110 nonpregnant adult patients with chikungunya virus infection, by presence or absence of sepsis or septic shock, University Hospital of Pointe-à-Pitre, Guadeloupe, French West Indies, January–November 2014*

Characteristic	Severe sepsis or septic shock, n = 25	No severe sepsis or septic shock, n = 85	p value†
Baseline characteristics			
Median age, y (interquartile range)	70 (59–77)	70 (59–78)	0.966
Male sex	17 (68)	45 (53)	0.252
Preexisting comorbid conditions			
Immune suppression	1 (4)	4 (5)	1
Diabetes mellitus	10 (40)	34 (40)	1
Chronic heart disease	6 (26)	14 (17)	0.374
Cerebrovascular disease	1 (4)	6 (8)	1
Chronic lung disease	2 (9)	1 (1)	0.124
Chronic liver disease	1 (4)	0 (0)	0.223
Chronic renal disease	3 (13)	6 (8)	0.414
Cancer	1 (4)	6 (7)	1
Charlson index, median (interquartile range)	4 (3–5)	4 (2–5)	0.579
McCabe class 1	9 (39)	21 (26)	0.296
Clinical symptoms, present on admission to hospital			
Arthralgia/arthritis	19 (91)	60 (80)	0.347
Headache	3 (17)	21 (30)	0.376
Fever	21 (84)	75 (90)	0.467
Myalgia	8 (44)	29 (45)	1
Cardiac manifestations	20 (80)	18 (22)	<0.001
Central nervous system manifestations	8 (32)	25 (30)	1
Respiratory manifestations	18 (72)	20 (24)	<0.001
Hepatic manifestations	7 (28)	6 (8)	0.012
Renal manifestations	17 (71)	15 (19)	<0.001
Organ failures, at any time of the course of the disease			
Cardio-circulatory failure	17 (68)	5 (6)	<0.001
Neurologic failure	3 (12)	3 (4)	0.129
Respiratory failure	14 (56)	7 (8)	<0.001
Liver failure	6 (24)	3 (4)	0.004
Renal failure	13 (52)	2 (2)	<0.001
Laboratory data, median (interquartile range)			
Whole leukocytes, day 1, G/L	8.10 (6.10–13.10)	5.80 (4.10–7.10)	0.004
Whole leukocytes, days 2–7, G/L	10.70 (3.40–15.00)	3.05 (2.35–7.00)	<0.001
Polymorphonuclear neutrophils, day 1, G/L	5.96 (4.48–11.02)	4.30 (2.82–5.91)	0.01
Polymorphonuclear neutrophils, days 2–7, G/L	8.73 (2.55–12.02)	1.80 (1.09–5.86)	<0.001
Hemoglobin, day 1, g/dL	12.1 (10.6–13.5)	12.5 (11.2–13.5)	0.796
Hemoglobin, days 2–7, g/dL	10.6 (7.9–11.7)	11.7 (10.3–12.9)	0.013
Platelets, day 1, G/L	139 (106–192)	176 (123–233)	0.063
Platelets, days 2–7, G/L	104 (59–189)	149 (108–200)	0.03
C-reactive protein, day 1, mg/L	46 (20–75)	40 (20–79)	0.856
C-reactive protein, days 2–7, mg/L	92 (41–204)	70 (33–106)	0.119
Lactate dehydrogenase, day 1, IU/L	606 (288–946)	310 (226–401)	0.007
Lactate dehydrogenase, days 2–7, IU/L	422 (346–1600)	363 (266–422)	0.05
Creatine kinase, day 1, IU/L	653 (304–1394)	264 (140–639)	0.08
Creatine kinase, day 2, IU/L	911 (357–3932)	727 (163–2642)	0.283
Aspartate aminotransferase, day 1, IU/L	60 (39–127)	33 (24–48)	0.001
Aspartate aminotransferase, days 2–7, IU/L	125 (36–626)	54 (35–104)	0.044
Alanine aminotransferase, day 1, IU/L	33 (24–57)	20 (15–31)	0.02
Alanine aminotransferase, days 2–7, IU/L	56 (28–499)	30 (19–61)	0.055
Creatinine, day 1, μmol/L	157 (114–343)	96 (77–134)	0.008
Creatinine, days 2–7, μmol/L	226 (123–570)	101 (76–141)	0.002
Outcome			
Death	12 (48)	2 (3)	<0.001

*Values are no. (%) except as indicated. All patients were hospitalized and had infection confirmed by reverse transcription PCR.
†p value denotes the comparisons between the 25 patients with severe sepsis or septic shock and the 85 patients with no severe sepsis or septic shock, based on nonparametrical tests.

mortality rate was significantly higher than that in patients without severe sepsis or septic shock (48% vs. 3%, p<0.001).

The following case report describes one of the 25 patients with severe sepsis or septic shock. The patient died of septic shock, which had no other identified cause but CHIKV infection.

An 85-year-old man with no prior medical history except treated hypertension developed an acute influenza-like syndrome. On day 2 of illness, a common form of CHIKV infection was diagnosed by his general practitioner, and the patient received treatment for his symptoms. On day 4, he was referred to a hospital emergency department because of

persistent high-grade fever. His hemodynamic condition was normal, and the diagnosis of CHIKV infection was maintained; however, because of elevated levels of whole leukocytes (total 40 G/L), polymorphonuclear neutrophils (37.5 G/L), and serum C-reactive protein (170 mg/L), blood and urine samples were collected for culture, and treatment with ceftriaxone was started. Approximately 12 hours after admission to the emergency department, the patient experienced onset of septic shock and died within 4 hours. All blood and urine cultures were negative for CHIV. A PCR test for leptospirosis also was negative. A CHIKV-positive RT-PCR test result was the only positive diagnostic test result obtained for this patient.

Conclusions

Although chikungunya usually has a mild course, severe life-threatening complications can develop during the acute phase of the disease (*6*,*7*). Previous studies indicate that the disease can be complicated by severe multiple organ failure and lead to death (*8*,*9*). Very recently, the first cases of severe sepsis and septic shock that could be attributed to CHIKV infection were reported (*10*,*11*). In some of these cases, acral skin necrosis was observed (*11*).

The replication of viruses, especially of the family *Herpetoviridae*, has been shown to occur frequently during the course of septic shock syndromes of bacterial origin, not only as a stress-induced reactivation but also as a superinfection causing additional morbidity (*12*). By contrast, cases of virus-triggered septic shock have been reported only rarely (*13*), although a recent cross-sectional study of septic shock syndromes in a pediatric population suggested that viruses might be the only etiology in up to 10% of cases (*14*). On the other hand, genuine acute severe viral infections might be complicated with a bacterial septic shock, which is well known to occur in cases of influenza but has also been reported in cases of arboviral diseases, such as dengue fever (*15*).

In our study, none of the 25 patients who had a positive CHIKV RT-PCR test result and a severe sepsis or septic shock syndrome early in the course of chikungunya had another organism identified as a potential cause of sepsis. This finding strongly suggests that CHIKV can, in rare cases, cause severe sepsis and septic shock syndromes, an observation that had not been reported until very recently. Additional studies are needed to identify any background characteristics that might be associated with the onset of severe sepsis or septic shock.

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Clinical, Virologic, and Epidemiologic Characteristics of Dengue Outbreak, Dar es Salaam, Tanzania, 2014

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We investigated a dengue outbreak in Dar es Salaam, Tanzania, in 2014, that was caused by dengue virus (DENV) serotype 2. DENV infection was present in 101 (20.9%) of 483 patients. Patient age and location of residence were associated with infection. Seven (4.0%) of 176 patients were co-infected with malaria and DENV.

Data are scarce on seroprevalence of dengue virus (DENV) in Tanzania. Cross-sectional studies conducted during 2007–2014 indicated that DENV seroprevalence ranged from <2% to >50%, depending on geographic area and epidemiologic characteristics of patients (*1*–*6*). *Aedes aegypti* mosquitoes, the main vector of DENV, are present throughout Tanzania (*7*), and the clinical course of DENV infection is greatly affected by previous exposure to different DENV serotypes (*8*). Investigation of DENV outbreaks might serve to define circulation of different serotypes and the best strategy to manage future outbreaks.

In 2014, a large dengue outbreak occurred in Dar es Salaam, Tanzania (*8*). We report the main findings of a study conducted there during the outbreak.

The Study

Ethical approval for the study was obtained from the National Health Research Ethics Sub-Committee of Tanzania (protocol no. NIMR/HQ/R.8a/Vol. IX/I733). Informed consent was obtained from all participants.

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The study involved the 3 districts of Dar es Salaam (Kinondoni, Ilala, and Temeke (Figure 1, panel A). All consecutive patients with fever (temperature >37.5°C) for <7 days who came to the outpatient department of 1 of 7 selected health facilities were tested for malaria by using a malaria rapid diagnostic test (mRDT) (SD Bioline Malaria Ag. Pf/Pan Test, Bioline, Gewerbestrasse, Switzerland) and for dengue by using a DENV rapid diagnostic test (dRDT) (SD Bioline Dengue Duo; Standard Diagnostics, Inc., Gyeonggi-do, South Korea).

DENV infection was defined as a positive result for DENV nonstructural protein 1 (NS1) or IgM against DENV detected by dRDT. Past DENV infection was defined as IgG against DENV alone detected by dRDT. Malaria infection was defined as a positive result by mRDT. Warning signs and severe dengue were defined according to guidelines of the World Health Organization (*9*). A structured interview was used to collect demographic, environmental, and clinical data. Molecular analyses were conducted at the National Institute for Infectious Diseases L. Spallanzani (Rome, Italy).

Virus RNA was extracted and 2 real-time reverse transcription PCRs (RT-PCRs), one specific for DENV serotypes 1, 2, and 3 and one for DENV serotype 4, were performed on dRDT-positive serum samples as described (*10,11*). For identification of DENV serotypes, a fragment spanning the E–NS1 gene junction was amplified as described (*12*). A complete envelope (E) gene sequence was obtained by using a One-Step RT-PCR Kit (QIAGEN, Hilden, Germany) and primers (primer sequences available on request). Sanger sequencing and phylogenetic analysis based on the nucleotide sequence of the E–NS1 region and a complete E gene sequence (Figure 2) were performed. A multiple logistic regression model with a backward procedure was used to determine a cutoff level of $p = 0.10$.

A total of 483 of 491 patients who came to health facilities and matched inclusion criteria were enrolled in the study. Among enrolled patients, 101 (20.9%) were positive for DENV infection, and 9 (1.9%) were positive for past DENV infection. Incidence of DENV infections peaked during mid-June and decreased toward the end of the month (Figure 1, panel B).

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[2]These senior authors contributed equally to this article.

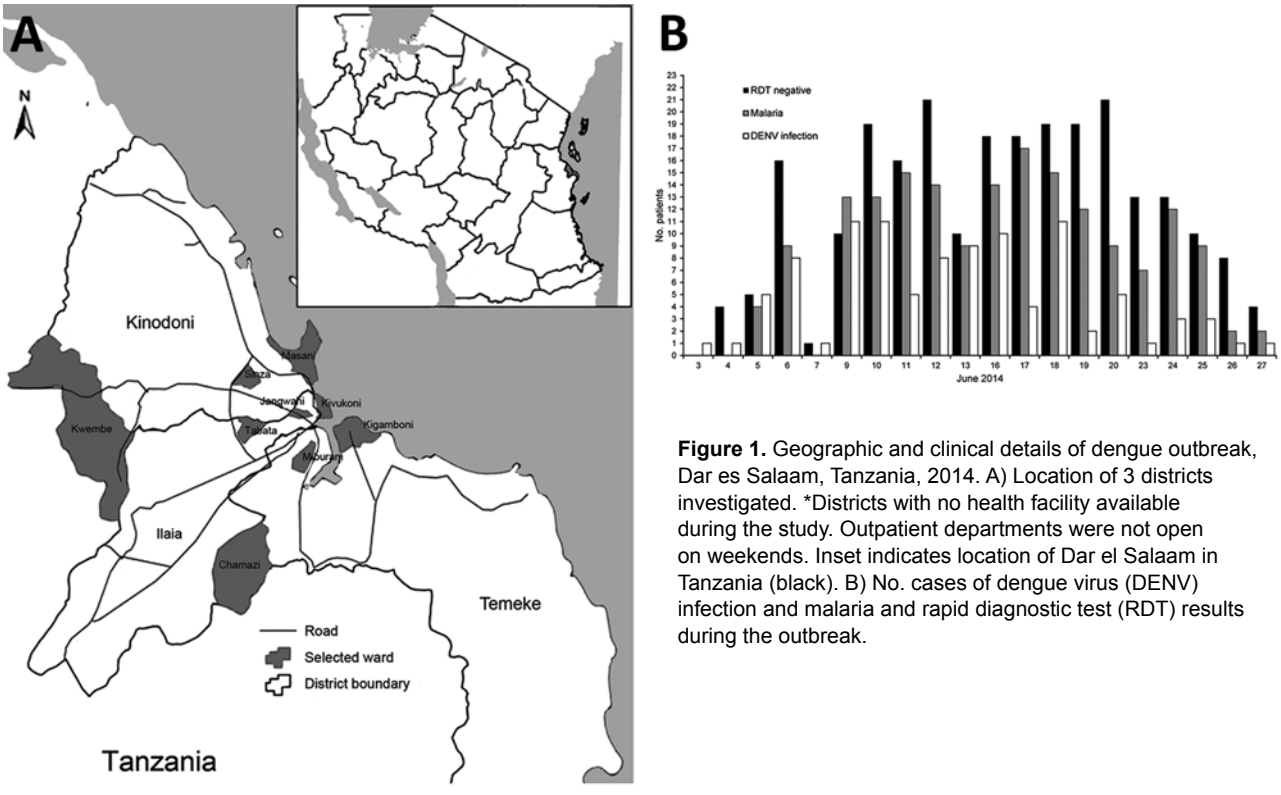

Figure 1. Geographic and clinical details of dengue outbreak, Dar es Salaam, Tanzania, 2014. A) Location of 3 districts investigated. *Districts with no health facility available during the study. Outpatient departments were not open on weekends. Inset indicates location of Dar el Salaam in Tanzania (black). B) No. cases of dengue virus (DENV) infection and malaria and rapid diagnostic test (RDT) results during the outbreak.

Univariate analysis showed that current DENV infection was associated with patient age (p<0.001), location of residence (p = 0.007), and employment status (p<0.001) (Table). Multivariate analysis showed that only age and location of residence were independently associated with current DENV infection. The risk for infection for patients >15 years of age was twice that for patients ≤15 years of age (odds ratio 2.54, 95% CI 1.10–5.87; p = 0.029). Patients who lived in Kinondoni were twice as likely to have a DENV infection than patients who lived in Temeke (odds ratio 2.83, 95% CI 1.44–5.56; p = 0.002).

Only joint and muscle pain were associated with DENV infection (p<0.001). Warning signs were more frequent in patients with DENV infection (42/101, 41.6%; p = 0.006). Three patients met criteria for severe dengue. Patients with DENV infection were more likely to be hospitalized (p<0.001) and to have used antimalarial drugs (p = 0.025). A total of 176 (34.6%) patients had a positive result for malaria by mRDT. Of these patients, 7 (4.0%) were co-infected with DENV (positive result for NS1), and 14 (8.0%) had a recent DENV–malaria coinfection (IgM or IgM/IgG positive). Sixty-two patients with DENV infection had positive results by PCR.

Sequence analysis of NS1–E junction gene fragments of DENV was performed for 8 randomly selected RT-PCR–positive samples (GenBank accession nos. KT288895–KT288902). Phylogenetic analysis of all sequences grouped them in a monophyletic cluster in the DENV serotype 2 cosmopolitan genotype. Further sequencing of the complete E gene was performed for 1 isolate (GenBank accession no. KT288902). Clustering with the DENV serotype 2 cosmopolitan genotype was confirmed (Figure 2). This analysis showed similarity of DENV in Tanzania with those from Asia isolated since 2001 and a strong phylogenetic relationship (99.8% identity) with a DENV variant isolated in Guangzhou, China, in 2013 (GenBank accession no. KJ807797).

Conclusions

We report DENV serotype 2 (Cosmopolitan genotype) as the causative agent of the dengue outbreak in Dar es Salaam in 2014. Sequence analysis showed that this virus from Tanzania had a strong phylogenetic relationship with virus strains from China, India, East Timor, and Singapore. These results indicate that the virus could have been recently introduced into Tanzania by travelers from Asia. This hypothesis is supported by the phylogenetic relationship with sequences obtained from isolates in Asia since 2001 and by reports of DENV-3 detection in the previous years in Zanzibar (*4,5*).

The incidence of DENV infection in Dar es Salaam (20.9%) is higher than incidences reported in recent studies of household participants in Angola (9% of recent infections) (*13*) and Kenya (13% of recent/current infections). This discordance might be related to different

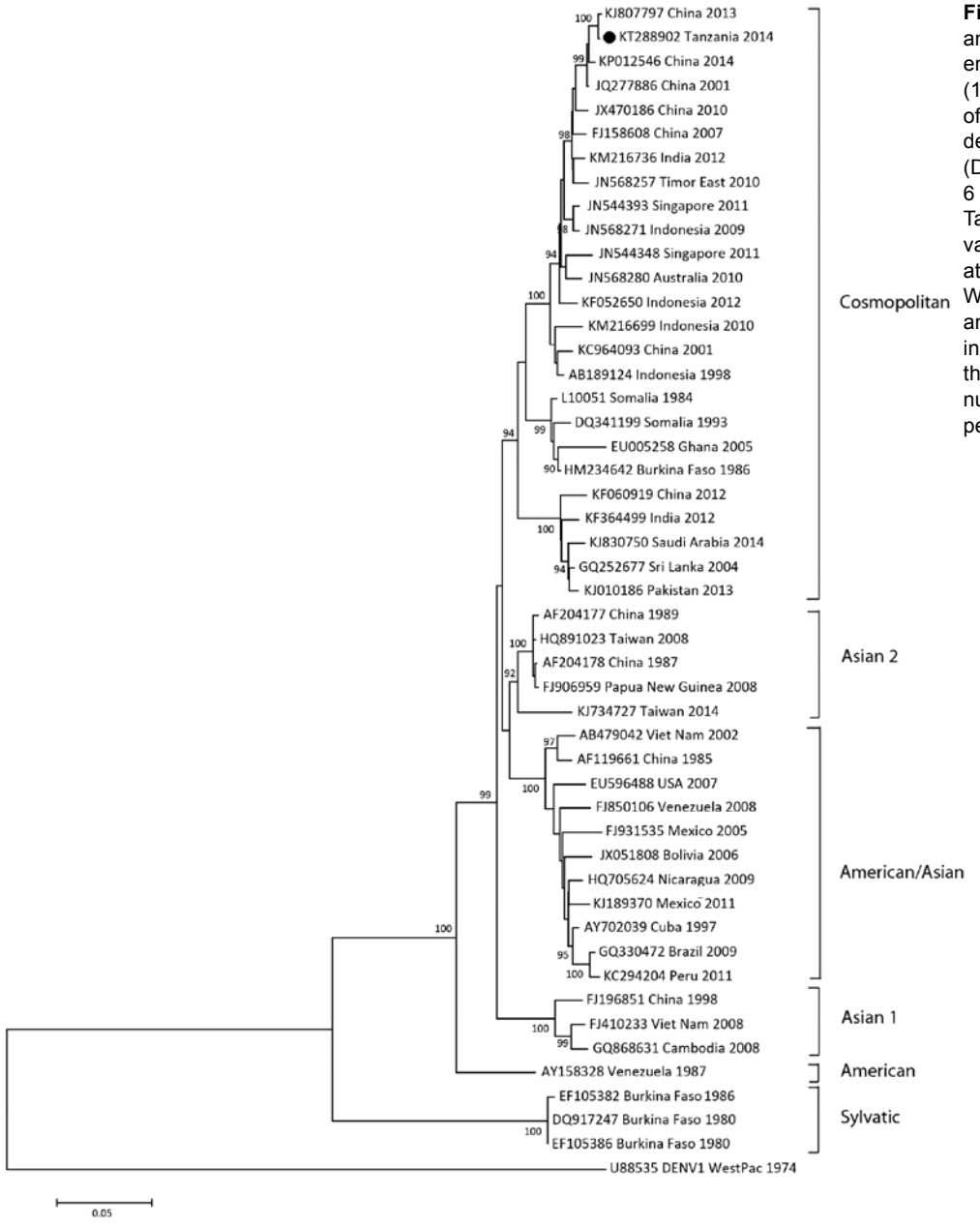

Figure 2. Phylogenetic analysis of complete envelope gene sequences (1,485 nt, position 937–2421 of strain KP012546) of 48 dengue virus serotype 2 (DENV2) strains representing 6 genotypes, Dar es Salaam, Tanzania, 2014. Bootstrap values (>90%) are shown at key nodes. DENV1 West Pacific was used as an outgroup. Solid circle indicates strain isolated in this study. Scale bar indicates nucleotide substitutions per site.

designs of these 2 studies. Our study was conducted with febrile patients attending health facilities and not in households. Moreover, the dRDT used in our study has a lower specificity than that of tests used in Angola and Kenya.

The higher incidence of DENV infection among elderly patients is not consistent with recent data for other disease-endemic regions (*13*) and might be related to possible recent introduction of the DENV serotype 2 in the area. The higher incidence reported in Kinondoni District could be related to the urban nature of the district.

As reported in Africa (*13*,*14*), no environmental or behavioral factors were associated with DENV infection. Malaria and DENV co-infection was present in 4% of patients, a find similar to that in a recent study in Ghana, where possible co-infection in was reported in 3.2% of children (*15*). As reported in Kenya (*14*), the high use rate (33.3%) for antimalaria treatment in patients with DENV infection during the current outbreak suggests a high level of underrecognition of dengue.

External validity of our results should be evaluated according to potential limitations of the study. First, the study used passive recruitment, which might have

Table. Characteristics of 483 patients tested for DENV infection during dengue outbreak, Dar es Salaam, Tanzania, 2014*

Characteristic	Current dengue infection, n = 101	No dengue, n = 382	Total, n = 483	p value
Sex				0.067†
M	65 (64.4)	207 (54.2)	272 (56.3)	NA
F	36 (35.6)	175 (45.8)	208 (43.7)	NA
Median age, y (IQR)	28 (21–37)	24 (13–35)	25 (14–36)	<0.001‡
Age, y, n = 481				<0.001†
≤15	12 (11.9)	117 (30.8)	129 (26.8)	NA
>15	89 (88.1)	263 (69.2)	352 (73.2)	NA
District				0.007†
Temeke	18 (17.8)	123 (32.2)	141 (29.2)	NA
Ilala	34 (33.7)	128 (33.5)	162 (33.5)	NA
Kinondoni	49 (48.5)	131 (34.3)	180 (37.3)	NA
Employed, n = 455	66 (69.5)	169 (46.9)	235 (51.7)	<0.001†
Water storage, n = 476	43 (43.9)	185 (48.9)	228 (47.9)	0.371†
Resting water, n = 481	48 (48.5)	179 (46.9)	227 (47.2)	0.773†
Persons in household, n = 474				0.284†
1–3	33 (33.3)	118 (31.5)	151 (31.9)	NA
4–6	41 (41.4)	185 (49.3)	226 (47.7)	NA
≥7	25 (19.2)	72 (19.2)	97 (20.5)	NA
Bed net use, n = 479	75 (75)	313 (82.6)	388 (81)	0.085†
Insecticide spraying in home, n = 479	20 (20)	87 (21)	107 (22.3)	0.528†

*Values are no. (%) except as indicated. DENV, dengue virus; NA, not applicable; IQR, interquartile range.
†By χ^2 test.
‡By Wilcoxon rank-sum test.

resulted in potential selection bias (only sicker patients who came to selected health facilities were analyzed). Second, the study had virtually no follow-up, which precluded any inferences about clinical outcomes of patients with severe dengue.

Despite these limitations, our study provides useful information on an underreported disease and on the molecular epidemiology of largest dengue outbreak reported in Tanzania. Results of our study can improve awareness of healthcare providers and demonstrate the feasibility of interventions to enhance diagnostic testing capabilities and specific surveillance systems. Resurgence of dengue in Tanzania should prompt implementation of population-based studies on differential diagnosis of acute febrile illness and surveillance systems based on syndromic approaches.

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Fatal Septicemia Linked to Transmission of MRSA Clonal Complex 398 in Hospital and Nursing Home, Denmark

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We describe 2 fatal cases of methicillin-resistant *Staphylococcus aureus* (MRSA) clonal complex 398 septicemia in persons who had no contact with livestock. Whole-genome sequencing of the isolated MRSA strains strongly suggest that both were of animal origin and that the patients had been infected through 2 independent person-to-person transmission chains.

Methicillin-resistant *Staphylococcus aureus* (MRSA) clonal complex (CC) 398 is associated with livestock and can spread to humans who have contact with animals (*1*,*2*). The percentage of persons infected with MRSA CC398 is increasing rapidly. In 2014, MRSA CC398 accounted for 43% of all cases of MRSA infection in Denmark (*3*). MRSA CC398 has not been thought to spread easily from person to person (*1*,*2*,*4*) and had been regarded as less virulent than other human MRSA strains (*2*,*5*). Recent studies have showed that MRSA CC398 is an increasing cause of colonization and infection among persons with and without livestock exposure in Germany, the Netherlands, and Denmark (*6*–*8*). However, the transmission route of MRSA CC398 of animal origin to persons with no reported contact with livestock is still unknown. Only a few small outbreaks of MRSA CC398 infection have been reported (*9*–*11*), but animal origin of the bacteria was not documented in those cases.

We describe 2 fatal cases of septicemia attributable to MRSA CC398 of animal origin in a hospital hemodialysis unit and a nursing home. Neither of the patients had any reported contact with livestock. The results of our investigation strongly suggest that transmission occurred through asymptomatic carriers in the 2 institutions.

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The Study

Transmission Chain 1

Patient 1

A 63-year-old man with diabetes and end-stage kidney failure had been receiving maintenance hemodialysis in the outpatient clinic at Odense University Hospital in Odense, Denmark, since 1997. A femoral–femoral bridge graft was used for vascular access. In November 2013, he was admitted to the hospital because of a fever he experienced during dialysis and inflammation around his bridge graft. Cultures of blood samples taken at admission grew MRSA CC398 *spa* type t011. The organism was also cultured from the patient's bridge graft and from a sample of joint fluid from his right shoulder. A transesophageal echocardiography revealed mitral valve endocarditis. Despite relevant treatment with vancomycin and rifampin and surgical debridement of his shoulder joint, blood cultures remained positive for MRSA until he died 3 weeks later. On inquiry by the staff, the patient had reported no previous history of MRSA infection or colonization and no direct or household-related contact with pigs.

Patient 2

Four months before patient 1's illness, MRSA CC398 *spa* type t011 had been cultivated from an infected decubitus ulcer of another patient who was receiving hemodialysis in the same outpatient clinic as patient 1. Subsequent MRSA screening revealed that patient 2 was a nasal and pharyngeal carrier. On inquiry by the staff, the patient reported no direct or household-related contact with pigs.

Transmission Chain 2

Patient 3

A 74-year-old nursing home resident had hemiparesis and recurrent aspiration pneumonia after an apoplectic insult. In April 2014, he was admitted to the hospital with severe pneumonia. On admission, he had sepsis. Blood cultures grew MRSA CC398 *spa* type t034, and the organism was found in a tracheal aspirate and from the area around a percutaneous gastrostoma tube. Despite relevant treatment with piperacillin/tazobactam, metronidazole, and vancomycin, the patient died from respiratory failure after 1 week. On inquiry by the staff, the patient and his attending daughter reported no direct or household-related contact with pigs.

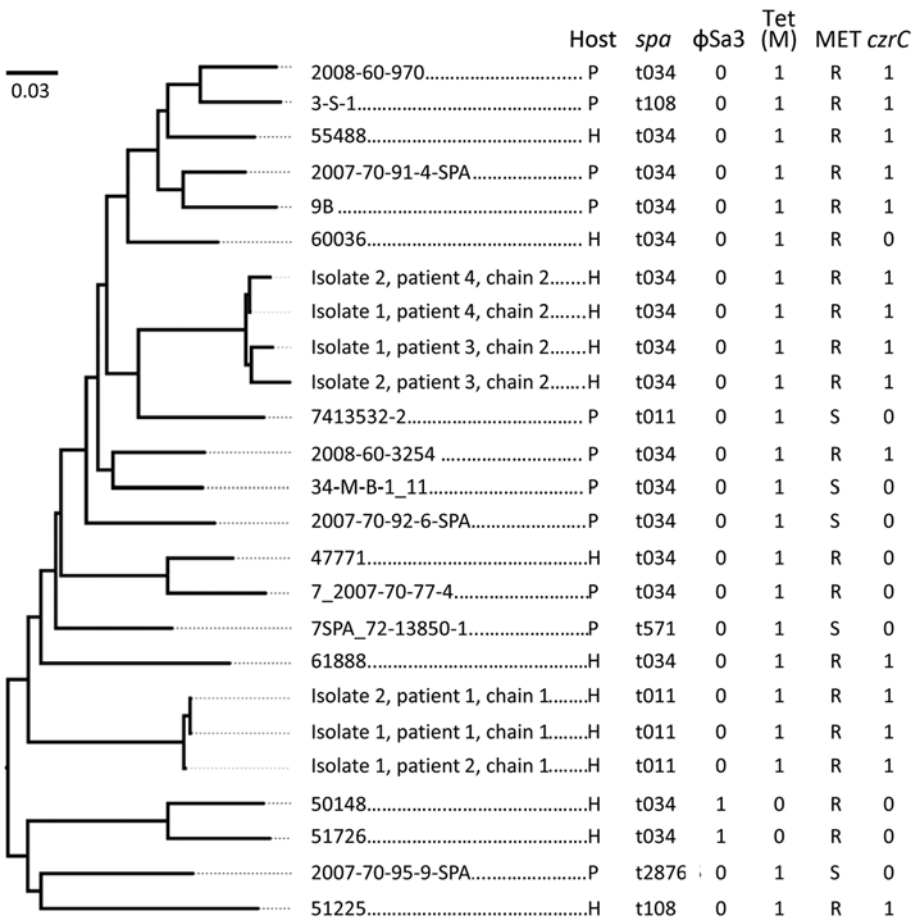

Figure. Phylogeny of methicillin-resistant *Staphylococcus aureus* (MRSA) clonal complex (CC) 398 isolates linked to fatal septicemia in a hospital patient and a nursing home resident in Denmark. Draft whole-genome sequencing was performed on 7 isolates from the 4 patients identified in the 2 transmission chains, and results were compared with similar genomic data for CC398-related MRSA and methicillin-sensitive *S. aureus* isolates obtained in Denmark during a previous study of isolates belonging to CC398 (*13*). Single-nucleotide polymorphism differences were identified, and a maximum-likelihood phylogeny was inferred from raw data by using the web tool CSI Phylogeny (https://cge.cbs.dtu.dk//services/CSIPhylogeny). The reference strain was S0385 (GenBank accession no. AM990992.1). The region of bp 12252–135180 was excluded from analysis because it contains the *spa* region and disrupts the phylogenic signal (*13*). Scale bar indicates substitutions per site. P, pig; H, human; MET, methicillin susceptibility; R, resistant; S, susceptible.

Patient 4

In July 2010, MRSA CC398 *spa* type t034 had been isolated from a bedsore of another patient living in the same nursing home as patient 3. In November 2013, the organism was isolated once more from the patient, this time from an indwelling urine catheter. No contact with pigs were reported on inquiry of the patient and her family. Patients 3 and 4 lived in the same wing of the nursing home and shared common facilities. After hospital staff recognized the transmission chain, all residents in this wing and all attached staff were screened for MRSA, but none tested positive.

All 4 of the affected patients lived in urban areas. None of them had any proximity to pig farms.

The MRSA CC398 isolates identified in the 2 transmission chains were *spa* types t011 and t034, which are common among livestock-associated MRSA strains (*12*). Draft whole-genome sequencing was performed on 7 isolates from the 4 patients in the 2 transmission chains, and results were compared with similar data for CC398-related MRSA and methicillin-sensitive *S. aureus* isolates from Denmark obtained during a previous study of the global dissemination of isolates belonging to this clonal complex (*13*). Sequence files for the individual isolates from patients are available at the European Nucleotide Archive (http://www.ebi.ac.uk/ena; accession no. PRJEB11281). On the basis of these data, a phylogenetic tree was constructed from single-nucleotide polymorphism differences in genome sequences of isolates from the 4 patients and from the isolates obtained during the previous study by using the S0385 complete genome sequence (GenBank accession no. AM990992.1) as reference (Figure). This analysis showed that the patient isolates within each transmission chain were closely related but that no close relation between the 2 chains existed. In addition, the isolates from both chains clustered among isolates previously found to be animal associated (clade IIa; *13*). Consistent with an animal origin, all isolates carried the *tet*(M) and *czrC* determinants (similar to most MRSA strains from clade IIa) and lacked the ΦSa3 phage (typical of the human clade I) (*13*).

Conclusions

We report 2 fatal MRSA CC398 infections after human-to-human transmission in institutional settings. Both patients had debilitating underlying diseases but were in a stable condition until the time of their infections. The sequence of events leaves no doubt that septicemia attributable to

MRSA CC398 was the cause of death in both cases. The CC and *spa* types of the isolates causing fatal infections were typical for MRSA isolates from pigs. Phylogenetic analyses of whole-genome sequences indicated that the human isolates from the 2 transmission chains were located in different clusters that intermingled with isolates from pigs. The detection of molecular markers associated with livestock origin further confirmed animal origin. The different *spa* types and the clustering of the MRSA isolates from the 2 chains clearly indicate 2 separate chains of infection.

None of the 4 patients described here had occupational or household contact with livestock. However, epidemiologic investigations and typing analyses strongly suggested that the 2 MRSA-infected patients could have acquired their infections from an asymptomatic carrier in the outpatient hemodialysis unit (transmission chain 1, patient 2) and the nursing home (transmission chain 2, patient 4). Transmission of MRSA CC398 in hospitals and institutions has been reported elsewhere, which underscores its potential to spread through person-to-person contact (*9–11*).

In conclusion, the organism implicated in these 2 fatal cases was by all accounts spread from person to person. These findings suggest that this clonal complex can be of high pathogenicity and is readily transmissible among humans.

This study was approved by the Data Protection Agency of the Region of Southern Denmark (reference no.14/30968) and the National Board of Health of Denmark (reference no. 3-3013-769/1).

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Malaria Hyperendemicity and Risk for Artemisinin Resistance among Illegal Gold Miners, French Guiana

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To assess the prevalence of malaria among illegal gold miners in the French Guiana rainforest, we screened 205 miners during May–June 2014. Malaria prevalence was 48.3%; 48.5% of cases were asymptomatic. Patients reported self-medication with artemisinin-based combination therapy. Risk for emergence and spread of artemisinin resistance among gold miners in the rainforest is high.

Malaria control programs on the Guiana Shield, a region of South America, are challenged by migrant populations looking for gold. Since 2008, the "Harpie" operation to control and reduce illegal gold mining activities has been conducted by French Armed Forces in French Guiana. Military deployments at illegal gold mining sites have resulted in several outbreaks and increased incidence of malaria in French forces (*1–4*), which suggests high transmission levels in those areas. Illegal gold mining sites are isolated places in the rainforest, far from health posts. The miners are usually hidden in the forest to avoid police controls, and they live in unsanitary conditions.

Although formal health monitoring is not carried out in these communities, the effects of infectious diseases are of concern. In 2013, a group of 34 illegal gold miners with severe diarrheic and respiratory symptoms were evacuated by plane from a health post to the reference regional hospital in Cayenne. The outbreak was attributed to co-infection with several parasitic, bacterial, or viral agents: seasonal influenza A(H1N1)pdm09, *Shigella flexnieri*, *Necator americanus*, *Leishmania* spp., *Streptococcus pneumoniae*, and *Plasmodium vivax* (*5*). All patients came from the illegal gold mining site of Eau Claire (3°36′00″N, 53°34′60″W) (Figure 1). Given these problems, French health authorities decided to provide primary medical care in the field and also to assess the sanitary situation in Eau Claire. We describe the results of the cross-sectional study conducted to assess the epidemiologic situation of malaria.

The Study

Because of violence and insecure conditions at illegal mining sites, French military health services conducted the mission. Twelve soldiers and military policemen accompanied the medical team to ensure their protection but also that of the miners' community. A primary care clinic and laboratory were set up under tents at the Eau Claire gold mining camp from May 28, 2014, through June 6, 2014. Active malaria screening was offered to every person who sought care for any reason.

Diagnostic tests associating the malaria rapid diagnosis test (RDT) (SD Bioline Malaria Ag *Pf*/Pan; Standard Diagnostics, Inc., Giheung-gu, South Korea) and thin blood films were performed in the field. Patients who had positive results of an RDT, thin blood film, or both received treatment.

Data were collected by physicians concerning each person's recent medical history, protection measures against mosquito bites, use of medications, and recent travel inside or outside of French Guiana. Patient anonymity was stringently respected; every patient was issued a unique identification number. Only verbal consent could be obtained to avoid references that might reveal the identity of undocumented persons engaged in illegal activities.

Dried blood spots were obtained on filter paper by fingerstick and packaged in individual plastic bags with a desiccant until processing. *Plasmodium* DNA was extracted subsequently and tested with a nested PCR targeting *P. falciparum* and *P. vivax* 18S rRNA genes,

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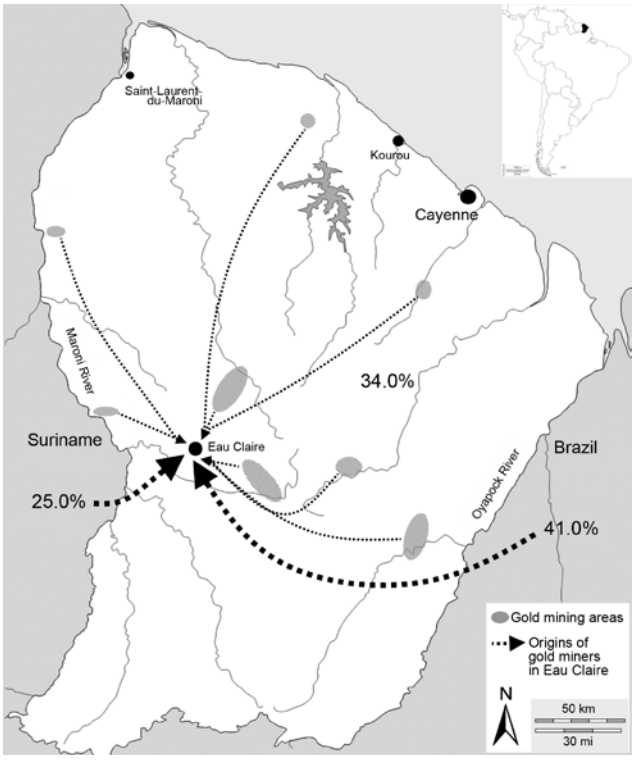

Figure 1. Origins of gold miners (N = 205) before they began to work in the illegal gold mining site of Eau Claire, French Guiana. Inset shows location of French Guiana in South America.

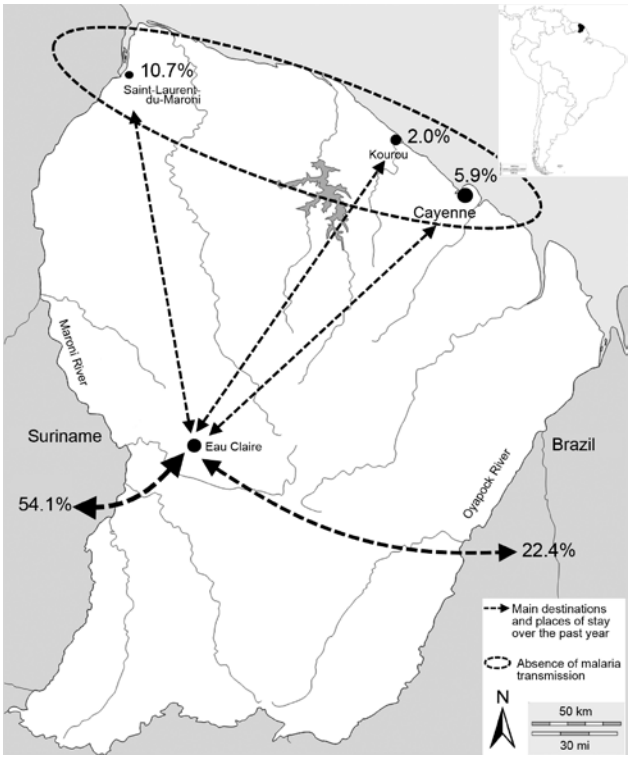

Figure 2. Travels of gold miners (N = 205) living in Eau Claire, French Guiana, 2013–2014. Inset shows location of French Guiana in South America.

according to the method of Snounou et al. (*6*). The propeller domain of *pfK13* gene was sequenced in *P. falciparum* isolates (*7*).

We defined *Plasmodium* infection as a positive RDT, thin blood film, or PCR result. Symptomatic *Plasmodium* infection was defined as a positive test result and fever (history of fever in preceding 24 hours and/or documented temperature ≥38°C during medical examination) and/or ≥2 of the following: nausea, vomiting, diarrhea, abdominal pain, anorexia, headache, or jaundice. Other *Plasmodium* infections were classified as asymptomatic.

Overall, 205 persons freely sought medical care and accepted malaria screening. The sex ratio was 2.0 (137 [66.8%] men; 68 [33.2%] women). Median age was 39 years (interquartile range [IQR] 32–46 years; range 20–63 years). The workers had been gold panning for a median time of 7 years (IQR 3–14 years; range 1 month–44 years) and on illegal gold mining sites in French Guiana for a median time of 4 years (IQR 1–8 years; range 1 month–25 years). Most (97.6%, 200) patients came from Brazil, 4 (2.0%) came from Suriname, and 1 (0.5%) came from Guyana. Before their arrival at Eau Claire, patients had lived in Brazil (41.0%, 84), Suriname (24.9%, 51), or at another illegal gold mining site (19/69 [33.7%] different sites throughout inland French Guiana and 1 unknown site) (Figure 1). During the previous year, 66.0% (135/205) persons had traveled to ≥1 area outside Eau Claire: 54.1% (111/205) to Suriname (among those, 60.0% had traveled there >2 times); 22.4% (46/205) to Brazil; and 18.5% (38/205) to the main cities in the malaria-free area along the French Guiana coast (Figure 2).

Table 1. Malaria and fever episodes reported by illegal gold miners, French Guiana, 2013–2014*

Episodes	No. (%) patients, N = 205
Malaria episode	
No	84 (41.0)
Yes	121 (59.0)
No. episodes	
1	27 (22.3)
2–4	48 (39.7)
>4	45 (37.2)
NA	1 (0.8)
Fever episode	
No	46 (22.4)
Yes	156 (76.1)
NA	3 (1.5)
No. episodes	
1	23 (14.8)
2–4	74 (47.4)
>4	57 (36.5)
NA	2 (1.3)

*NA, no answer.

Table 2. Number of positive parasite carriers by *Plasmodium* species according to diagnostic method, French Guiana, 2013–2014

Test	No. (%) single infections, n = 73		No. (%) mixed *P. falciparum*/*P. vivax* infections, n = 26	No. (%) total infections, n = 99	% Prevalence/ test, N = 205
	P. falciparum, n = 44	*P. vivax*, n = 29			
RDT*	23 (52.3)	8 (27.6)	8 (30.8)	40 (40.4)	19.5
Thin blood film	16 (36.4)	10 (34.5)	6 (23.1)	32 (32.3)	15.6
RDT and thin blood film	14 (31.8)	8 (27.6)	4 (15.4)	26 (26.3)	12.7
Cumulative RDT/thin blood film	25 (56.8)	10 (34.5)	10 (38.5)	46 (46.5)	22.4
PCR	42 (95.5)	28 (96.6)	26 (100.0)	96 (97.0)†	46.8†

*Malaria rapid diagnostic test: SD Bioline Ag *Pf*/pan (Standard Diagnostics, Inc., Giheung-gu, South Korea).
†In 3 cases (2 *P. falciparum* infections, 1 *P. vivax* infection) PCR results were negative, but results of thin blood films were positive.

Of the 205 patients, 156 (76.1%) reported ≥1 fever episode and 131 (63.9%) reported several (2–≥4) episodes; 121 (59.0%) reported ≥1 malaria episode and 93 (45.4%) several episodes (Table 1). Self-medication with antimalarial drugs was reported by 120 (58.5%) patients, of whom 118 (98.3%) had reported malaria episodes in the past year. Artemisinin-based combination therapies (ACTs) were mainly used: dihydroartemisinin/piperaquine/trimethoprim (Artecom; Chongqing Tonghe Pharmaceutical Co. Mingshan Town, China) by 79 (63.7%) and artemether-lumefantrine (Coartem; Novartis Pharmaceuticals Corp. Basel, Switzerland) by 32 (26.7%). Chloroquine was also used alone or with ACTs by 13 (10.8%) patients. The medication schedules used were not clearly identified, but 53 (44.2%) patients took drugs for 1 or 2 days only. Nets were used by 37 (18.0%) and mosquito repellents by 41 (20.5%) of the 205 patients.

The overall prevalence of malaria infection was 48.3% (99/205). *P. falciparum* and *P. vivax* single infections accounted for 44.4% (44/99) and 29.3% (29/99) cases, respectively, and mixed infection with *P. falciparum* and *P. vivax* for 26.3% (26/99) (Table 2). RDTs, thin blood films, and PCR were positive for 40.4% (40/99), 32.3% (32/99), and 97.0% (96/99) of patients classified as positive for malaria, respectively. Asymptomatic infections accounted for 48.5% (48/99) of cases. Low parasitemia levels were systematically observed. Only 1 person had a parsitemia level >1%, and no differences in level were found between symptomatic and asymptomatic infections. The propeller region of the *pfK13* gene was successfully sequenced in 26 *P. falciparum* isolates without any mutation detected.

Conclusions

During the 10 days of field work, almost all inhabitants of the gold mining site sought medical care. The high prevalence of malaria and asymptomatic *Plasmodium* infections observed confirms that malaria is hyperendemic there (*8*). Because of the mobility of gold miners within French Guiana, malaria also could be highly prevalent among persons at other illegal gold mining sites where competent vectors exist (*3*). This hypothesis is strengthened by the recurrent malaria outbreaks experienced by French forces involved in operations to control illegal gold mining, particularly in the center of the region (*3*).

Systematic self-medication by patients using ACTs without following a full course of treatment is a serious risk for emergence of resistance to artemisinin and associated drugs (*9*). The high price of ACTs in the field (2 g gold) was the primary reason patients gave for not completing treatments. The mobility of gold miners raises 2 issues: the reintroduction of the disease in malaria-free areas (*10*) and the spread of antimalarial drug resistance if the disease emerges (*11*). In 2013, parasite persistence on day 3 after treatment with Coartem was described in Suriname, and most participants in that study had worked in gold mines in French Guiana (*12*,*13*). In Guyana, *pfK13* C580Y mutants obtained from samples in 2010 were recently reported (*14*). These results may be viewed as ominous. Collaboration between countries of the Guiana Shield to control malaria among mobile populations is urgently needed (*15*).

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Linkage to Care for Suburban Heroin Users with Hepatitis C Virus Infection, New Jersey, USA

Eda Akyar, Kathleen H. Seneca, Serra Akyar, Neal Schofield, Mark P. Schwartz, Ronald G. Nahass

We identified a 41.4% prevalence of hepatitis C virus, absence of HIV, and unexpectedly high frequency of hepatitis C virus genotype 3 among suburban New Jersey heroin users 17–35 years of age during 2014–2015. Despite 2 clinicians prepared to engage these users, few were successfully linked to care and treated.

Hepatitis C virus (HCV) infection is a major public health issue. Although persons born during 1946–1964 represent most of the population with chronic HCV infection, young persons (17–35 years of age) who inject drugs (PWID) now make up the second wave of HCV infection. Up to 90% of all new HCV infections worldwide are attributed to injection drug use; at least 75% of new HCV infections in the United States result from injection drug use (*1*,*2*). In PWID, the prevalence of HCV is 60%–80% (*3*). Often, early age prescription opioid abuse leads to injection drug and heroin use (*2*). Escalating injection drug and heroin use has been associated with increasing HCV infection among young persons (*2*). An estimated 45% of young PWID in the United States are infected with HCV; the annual incidence is 8%–25% (*2*,*4*,*5*).

During 2006–2012 the incidence of acute HCV infections increased significantly among young people in nonurban areas of the United States (*2*). Among those persons, a 13% annual increase of acute HCV infection was reported in nonurban counties, a 170% increase over the course of 6 years (*2*). Young suburban heroin users have been described as the second wave of HCV infection in several US states, including Massachusetts and New York (*5*). New Jersey was not part of those initial reports. Our objective was to characterize HCV infection among young suburban heroin users in New Jersey and to evaluate linkage to care among this population.

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The Study

During October 1, 2014–June 9, 2015, Princeton House, a psychiatric facility in suburban New Jersey with an active opioid detoxification program, instituted a new HCV screening program. As part of the standard of care, patients admitted for heroin detoxification were tested for HIV, HCV, and hepatitis B virus infections. The average length of stay for opioid detoxification at Princeton House is 6 days. All patients positive for HCV antibody were clinically evaluated. HCV viral load and reflex genotypes (GTs) were obtained. Follow-up visits at Princeton House before discharge were performed by one of the authors (K.H.S. or R.G.N.) to counsel patients on results and the disease and to link patients to care by providing directions and appointments to HCV caregivers near patients' homes.

A total of 861 unique patients from 10 of 21 New Jersey counties were tested for HCV antibody; 374 (43.4%) were positive. Most (573 [66.6%]) patients were 17–35 years of age. Of those, 237 (41.4%) were HCV antibody positive. From this population, 187 patients were further evaluated; 50 patients refused evaluation or were discharged before evaluation. Women constituted 52.4%. Races and ethnicities were 173 non-Hispanic white, 2 non-Hispanic black, 4 Hispanic, and 8 other.

HCV viral load was obtained for 172 (92.0%) of the 187 patients; 15 patients were missed or not properly collected. For 32 (18.6%) patients, viral load was undetectable. HCV GTs were obtained from 102 patients: 64 (62.7%) were GT1a, 3 (2.9%) were GT1b, 8 (7.8%) were GT1 undefined, 1 (1.0%) was GT2, and 26 (25.5%) were GT3. Eight patients were identified with acute HCV. All patients who were HCV antibody positive were HIV antibody negative.

Of the 187 patients, 16 (8.6%) had outpatient follow-up appointments, and 3 (1.6%) started oral, direct-acting antiviral treatment. Two of the 3 patients failed to adhere to treatment regimen. One of the 16 patients spontaneously cleared infection before drug-treatment initiation. Two other patients returned for treatment but were denied prescriptions by insurance; all others failed to return for continued care.

Conclusions

Our study indicates that HCV was highly prevalent in young suburban heroin users attending an acute detoxification program that serves a wide geographic area,

suggesting that New Jersey is participating in the second wave of HCV infection. Our study highlights the challenges of linking young PWID in suburban areas to care despite the effort of 2 clinicians with extensive HCV experience to engage patients in the care cascade. That most patients were women (52.4%) and non-Hispanic white (92.5%) probably reflects the demographic of persons seeking detoxification from heroin and coincides with demographics of other reports of young nonurban PWID in the United Sates (*2*).

The 25.5% (95% CI 17%–34%) prevalence of GT3 among this population of young suburban heroin users is more than twice the national average of 12% (*6*). This pattern of distribution suggests a closed network of injection drug users engaging in risky behavior that leads to HCV transmission.

Although HCV screening was easily attainable in Princeton House, linking patients to care was a challenge (Figure). Even with encouragement, only 16 (8.6%) patients returned for in-office follow-up visits, and 2 started treatment. Patient follow-up after patients left Princeton House was a logistical challenge because of patient relocation and availability of transportation and communication. The program of acute detoxification at Princeton House simply withdrew patients from heroin under direct observation. Long-term patient management required additional treatment for patient addiction upon discharge, which was difficult to achieve. We were also limited by the absence of a care coordinator to assist with the linkage to care effort. Finally, some patients who came for outpatient treatment were denied treatment by payers because of the requirement of a clean drug test before treatment initiation or because patients did not have advanced liver disease, defined as Stage 3 or 4. All create additional complexity, which delays treatment. Because we did not formally assess patients' psychosocial circumstances, our considerations for the reasons for failure to link to care are speculative and are currently being studied. We believe that difficulty in linkage to care represents an area where greater support will be critical, such as through the use of case management, as was done with HIV care under the Ryan White program (*7*).

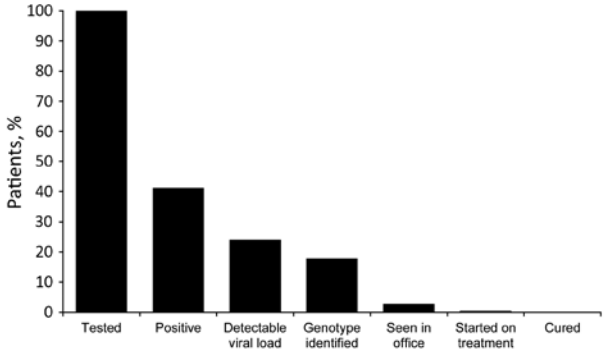

Figure. Cascade of care for suburban heroin users 17–35 years of age, New Jersey, October 1, 2014–June 9, 2015.

Also noteworthy is the absence of HIV in a population with a high percentage of injection drug use in a state with a high prevalence of HIV. Indiana's recent outbreak of HIV among young heroin users increases concern for the establishment of HIV in this network (*8*). To achieve success similar to that of HIV treatment in PWID, a coordinated program that includes committed case management services to help PWID navigate the complexity of accessing and maintaining treatment is likely to be needed. Further study to explore this and the ability to successfully treat this population is crucial to address the national HCV infection epidemic.

Finally, a misconception exists that PWID are poor candidates for treatment because of ongoing drug use, possible reinfection, and possible concomitant psychiatric or medical disorders (*9*). However, recent reports suggest that such patients can be successfully treated with newer therapies (*10*). Success in reducing HIV transmission among PWID during the past decade provides evidence that infected drug users can achieve adherence levels similar to persons who do not use drugs (*9*,*11*). The potential for treatment as prevention was discussed by Hellard et al., who calculated the minimum number of patients within a network needed to be treated to reduce or eliminate transmission (*10*). Given the availability of easy-to-use therapy that is curative, linkage to care and treatment of HCV-infected PWID may be an important public health effort to prevent the continued spread of HCV. Further study is needed to identify predictors for successful linkage to care.

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COMMENTARY

Threat from Emerging Vectorborne Viruses

Ronald Rosenberg

The earliest members of genus *Homo* were surely bedeviled by blood-feeding arthropods, some of which doubtless carried zoonotic pathogens. However, the phenomenon of vectorborne human epidemic disease began only after humans began building settlements 15,000 years ago (*1*). Settlements offered pathogens not only host density but also opportunities for their vertebrate reservoirs and arthropod vectors to cohabit with us. Epidemic *Yersinia pestis* (the Medieval Black Death) was only possible because black rats (*Rattus rattus*), the host of the vector flea, had become extraordinarily successful at living off human garbage and nesting in our buildings.

Two of the most important malaria vectors in the world exploit human activity to proliferate. Immature forms of *Anopheles gambiae* mosquitoes in Africa and *An. dirus* mosquitoes in Southeast Asia thrive in the small puddles (water-filled footprints, tire ruts, borrow pits, and drainage gullies) created around villages. Best adapted of all are *Aedes aegypti* mosquitoes, the cosmopolitan vector of epidemic yellow fever, dengue, chikungunya, and Zika viruses. Their ecologic niche is nearly ours. These mosquitoes lay eggs in artifacts: water storage jars, roof gutters, flower pots, dog dishes, even upturned bottle caps. Their cognate species, *Ae. albopictus*, is only slightly less versatile, having an attraction to discarded tires. Evolution of blood-feeding arthropods to our changing environment and evolution of some zoonoses to exploit this advantage are major links in the emergence of obscure pathogens into epidemic threats and is a timely subject for this issue of Emerging Infectious Diseases.

Persistence of human yellow fever, the seeming inexorable expansion of dengue, and the surprising, explosive spread and severity of first chikungunya virus and now Zika virus bear testament to the threat posed by habituated *Aedes* species. Since its arrival in the Western Hemisphere ≈1 year ago, Zika virus, which had previously been associated with a clinically mild and inconsequential illness, is now increasingly suspected of being the cause of an alarming epidemic of neurologic birth defects and Guillain-Barré syndrome in tropical regions. Zika virus, the subject of several articles in this issue, reminds us of some of the impediments to responding to emerging vectorborne pathogens.

First, Zika virus belongs to the most prevalent class of emerging pathogens, the zoonotic single-stranded RNA viruses, which have mutation rates as high as 1 base/10^4 bases each replication. The chikungunya pandemic that began 10 years ago was fueled in part by a single, nonsynonymous base change that enabled that alphavirus to replicate more efficiently in *Ae. albopictus* mosquitoes (*2*).

Second, conditions enabling transition from vectorborne animal-to-animal transmission to arthropod-mediated human-to-human transmission are poorly understood. Like dengue virus, another flavivirus, Zika virus was likely originally a pathogen of subhuman primates. Between its discovery in a sentinel macaque in Uganda in 1947 and the first recorded epidemic 60 years later in Yap, Federated States of Micronesia, only 14 human cases had been reported, all from Africa and Asia (*3*).

Third, the pathogenicity and transmission dynamics of vectorborne zoonotic pathogens are much more complex than those of directly communicable pathogens. It is not yet known if Zika virus will find sustaining, nonhuman hosts in the Western Hemisphere, as has yellow fever virus, or how wide the range of vector species will be. Pathogenicity and transmission dynamics will be factors in determining where Zika virus will become endemic and what will be the most suitable methods of control.

Fourth, accurate diagnosis is key to surveillance and response. It might seem as if Zika virus sprang from nowhere, but almost certainly it must have been infecting many more humans in Africa and Asia than we had been aware. Our ability to serologically diagnose infections with emerging arboviruses is often compromised by close antigenic relationships within virus families. Zika, dengue, West Nile, and yellow fever viruses can co-circulate, not only among themselves, but possibly with unidentified or poorly characterized flaviviruses. The limitations of current diagnostics are a primary reason why the association between Zika virus and birth defects remained speculative so long.

Fifth, vector control is a force multiplier that can reduce the risk from many viruses that would require the development of individual vaccines. However, insecticide resistance and application problems greatly impede effective implementation.

The best defense is preventing a problem from growing into a threat. Fewer than 20 of the 86 known pathogenic arboviruses can be considered major causes of human disease, and 3 of these, West Nile, chikungunya, and Zika viruses, have emerged from relative obscurity within only the past 20 years (*4*). At least another 200 cataloged arboviruses whose relationship to human disease is unknown have been isolated from arthropods or animals.

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The discovery of 3 highly pathogenic tickborne viruses in China and the United States during the past 5 years (*5*–*7*) underscores how unrepresentative even that large number might be. It is unrealistic to characterize each of these viruses. Besides needing better methods of vector control, we need a strategy for preemptively identifying arboviruses with the potential for emergence and to devote resources to better understand their transmission dynamics, their endemicity, and accurate diagnosis.

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Reactivation of Ocular Toxoplasmosis in Non-Hispanic Persons, Misiones Province, Argentina

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To the Editor: Ocular toxoplasmosis (OT), caused by the parasite *Toxoplasma gondii*, is known to be a major health problem in South America, especially in Colombia and Brazil (*1*–*3*). The highest prevalence of OT has been reported in Erechim, Rio Grande do Sul, Brazil, a state that borders Uruguay and Argentina, where a representative population-based household survey showed that 17.7% of 1,042 adults examined had OT (*1*). For comparison, it is estimated that ≈2% of *T. gondii*–infected persons in the United States manifest OT (*4*). It is still unclear whether the high rate of OT in South America is attributable to host or parasite genetic factors or differences in exposure rate (*5*,*6*).

In the province of Misiones in Argentina, the prevalence of OT in patients seeking care in an ophthalmic office is also high and has been documented as high as 20% (*7*). The area was mostly settled in the early 20th century by non-Hispanic European immigrants from Germany and Slavic countries who arrived through Southern Brazil.

We explored the relationship between ethnic origin and frequency of reactivation toxoplasmic retinochoroiditis (RTR) in patients who sought care in a private secondary care eye clinic in Oberá, Misiones, Argentina, during February 2004–May 2014. All patients with uveitis were examined by a single uveitis specialist (M.R.), who performed complete ophthalmological examinations, including visual acuity, anterior biomicroscopy, tonometry, and indirect ophthalmoscopy. Study inclusion criteria were presence of RTR in 1 or both eyes and specific *T. gondii* IgG in blood serum samples. A minimum of 3 months with no signs of intraocular inflammation was required to differentiate chronic active retinochoroiditis from 2 consecutive episodes of RTR.

The study included 112 nonimmunosuppressed patients with RTR. The patients completed a questionnaire including demographic data for the patient, as well as the first and last names and countries of origin of parents, grandparents, and great-grandparents. Informed consent was obtained from all participants and the study was approved by the human subjects review committee of Misiones Province.

The patients were divided into 4 groups (Table): 1) patients reporting ≥1 ancestor born in Spain were considered Hispanic (n = 29); 2) patients without Hispanic ancestry who had ≥1 ancestor born in Poland, Ukraine, Russia, or Belarus, and who spoke Polish, Ukrainian, or Russian were considered Slavic (n = 28); 3) patients without Hispanic or Slavic ancestry who had ≥1 ancestor born in Germany or the Austro-Hungarian Empire and who spoke German were considered Germanic (n = 46); and 4) patients who did not fulfill the criteria of any of the above-mentioned groups were designated as others (n = 9). All patients had RTR at baseline; 28 had ≥1 more RTR episode during the follow-up period (Table). Multiple logistic regression analysis, in which Hispanic patients were used as a reference group, showed that Germanic and Slavic patients had a higher risk for reactivation during the follow-up period, but the odds ratio was significant only for Slavic patients after adjustment for rural dwelling.

More than 95% of *Toxoplasma gondii* strains in Europe belong to the clonal type II lineage, whereas strains from

Table. Demographic baseline characteristics of patients with reactivation of OT and multiple logistic regression analysis of the association between ethnic origin and risk for RTR during follow-up, Misiones province, Argentina, 2004–2014*

Demographic and follow-up data	Ethnicity			
	Hispanic, n = 29	Slavic, n = 28	Germanic, n = 46	Other, n = 9
Demographic data				
Male sex†	13 (44.83)	14 (50)	20 (43.48)	5 (55.56)
Median age, y (range)†	31 (6–67)	32.5 (14–70)	31 (6–71)	29 (6–68)
Rural dwelling†	18 (62.07)	23 (82.14)	30 (65.22)	5 (55.56)
Follow-up				
Length, median mo†	37	42	38.5	27
≥1 reactivation toxoplasmic retinochoroiditis	4 (13.79)	10 (35.71)	12 (26.09)	2 (22.22)
OR (95% CI)	Reference	3.47 (0.93–12.85)‡	2,2 (0.63–7.65)†	1.78 (0.27–11.86)†
OR adjusted for rural dwelling (95% CI)	Reference	4.07 (1.05–15.68)§	2.2 (0.65–8.01)†	1.73 (0.26–11.64)†

*Data are no. (%) patients, unless otherwise indicated. Analysis was performed using IBM SPSS Statistics (IBM, Armonk, NY, USA). p values <0.05 were considered significant. OR, odds ratio; OT, ocular toxoplasmosis; RTR, reactivation toxoplasmic retinochoroiditis.
†p>0.05.
‡p = 0.06.
§p = 0.04.

South America are genetically divergent and diverse (*6*,*8*). We hypothesize that the European population is poorly adapted to South American strains and therefore more susceptible to OT. If this hypothesis is true, Native Americans who had a long history of exposure to atypical strains from South America should be more resistant to OT. This hypothesis is reinforced by a recent survey conducted among Mbyá-Guarani Indians, who had a serologic prevalence of toxoplasmosis 70%, but only 3.5% of them had toxoplasmic retinochoroidal lesions (M. Rudzinski, unpub. data).

Argentineans have a large incidence of European genetic heritage in their Y-chromosomal and autosomal DNA, but ≈50% of their mitochondrial gene pool is of Native American ancestry (*9*). The amount of admixture between Europeans and Native Americans with inheritance of resistance genes to OT from Native Americans may explain the difference of susceptibility to RTR between Hispanic and non-Hispanic Europeans. Admixture events between Europeans and Native Americans mainly involved Hispanics whose migration to Argentina started in the 16th century and continued until the mid-20th century. Persons in Argentina who have Spanish surnames can carry as much as 80% Native American genetic ancestry (*9*,*10*). In contrast, the Slavic and German Europeans migrated to Misiones only during a large surge of European immigration between 1890 and 1950, and did not have substantial admixture with Native Americans. Despite the fact that this study was not a random or representative sample of all ethnic groups in Argentina and the Native American mixture of the patients was not known, and environmental and dietary influences were not examined, our results suggest host genetic factors as determinants of disease severity in OT.

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Tropical Islands as New Hubs for Emerging Arboviruses

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To the Editor: The outbreaks of dengue virus (DENV), chikungunya virus (CHIKV), and Zika virus infection that occurred on islands in the Indian Ocean, the Pacific, and the Caribbean over the past decade have demonstrated the potential of these arboviruses to pose a global public health threat. All 3 viruses were first isolated in the mid-20th century in either African or Asian countries; however, until 2005, only DENV (family *Flaviviridae*, genus *Flavivirus*) was considered a global public health concern (*1*).

In 2005, CHIKV infection, which typically manifests as fever, joint pain, rash, and polyarthralgia, emerged on islands in the Indian Ocean. During the next 10 years, CHIKV (family *Togaviridae*, genus *Alphavivirus*) caused several outbreaks in the Indian subcontinent, Asia, and Central Africa, and autochthonous transmission was reported in Europe (*2*). In 2011, CHIKV appeared for the first time in the Pacific region; 2 years later, it had expanded throughout the region (*3*). At the end of 2013, CHIKV

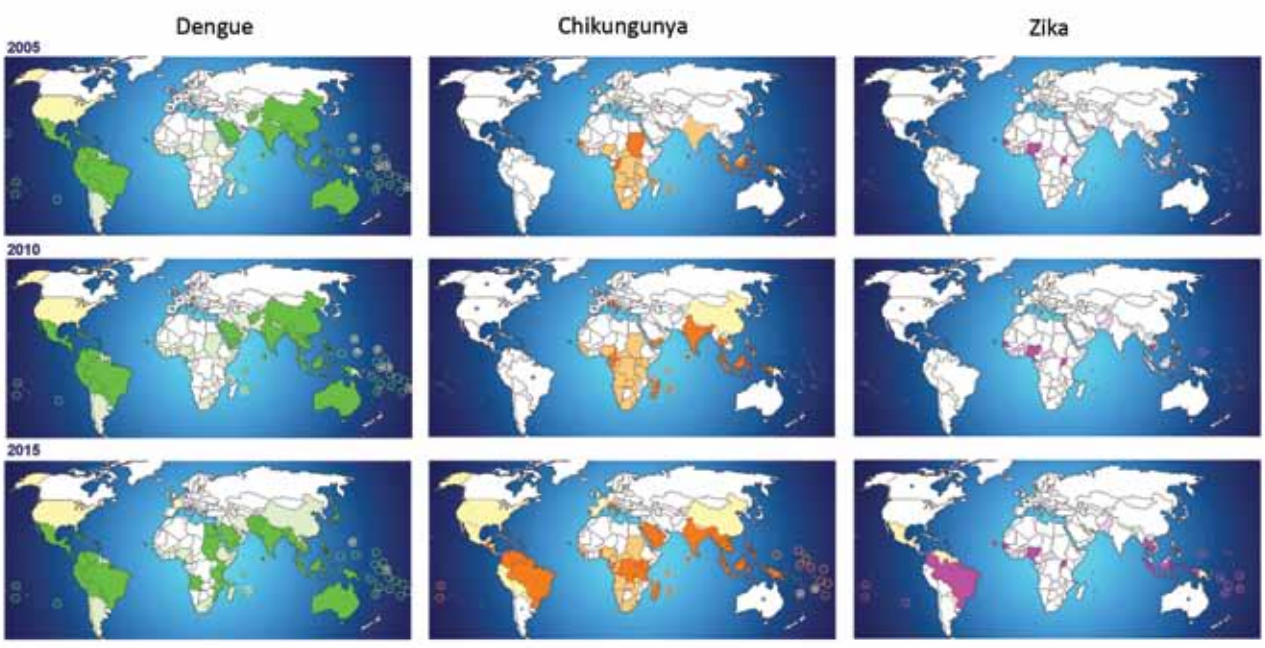

Figure. Areas affected by dengue, chikungunya, and Zika viruses, worldwide, 2005, 2010, and 2015, illustrating the evolution of the geographic distribution of these viruses over the past decade (*1*–*5*,*7*). Light shading/circles indicate countries with endemic transmission; dark shading/circles indicate countries with outbreaks recorded during the previous 5 years; dots indicate imported cases in countries without autochthonous transmission; stars indicate countries with reported autochthonous transmission.

emerged in the Caribbean and subsequently spread to the continental Americas, resulting in 1,726,539 suspected and 60,746 laboratory-confirmed CHIKV infections in the region as of December 18, 2015 (http://www.paho.org/hq/index.php?option=com_docman&task=doc_download&Itemid=&gid=30198&lang=en).

In 2007, Zika virus (family *Flaviviridae*, genus *Flavivirus*), which typically manifests as fever, joint pain, rash, and conjunctivitis, emerged for the first time outside Africa and Asia, in Yap State in Micronesia. Six years later, the virus caused a large outbreak in French Polynesia and then spread to other Pacific islands (*3*). In May 2015, autochthonous cases of Zika virus infection were confirmed in Brazil. By the end of the year, Brazil had declared an outbreak, and the virus had spread to several neighboring countries (http://www.paho.org/hq/index.php?option=com_docman&task=doc_download&Itemid=&gid=30198&lang=en).

The emergence of CHIKV and Zika virus in the Indian Ocean, the Pacific, and the Caribbean might result from multiple drivers. One factor is the presence of competent vectors, including the widely distributed *Aedes aegypti* and *Ae. albopictus* mosquitoes, but also endemic *Aedes* species that might serve as additional vectors, such as *Ae. hensilli* mosquitoes in Yap State (*4*). Small tropical islands also offer contexts conducive to mosquito proliferation and disease transmission; most meet the criteria to be listed as Small Islands Developing States and territories (SIDS) in the United Nations' framework of programs of action for sustainable development (http://www.un.org/documents/ga/conf167/aconf167-9.htm). SIDS are characterized by environments that are particularly sensitive and prone to natural disasters, populations that often lack safe water supplies and sanitation, and local governments that have limited resources to implement vector control and manage outbreaks. The increasing volume of travel between SIDS and continental regions where CHIKV and Zika virus are endemic has facilitated the spread of these viruses to previously unexposed populations.

Recent outbreaks of chikungunya and Zika have led to unexpected observations regarding the virulence and epidemic potential of such viruses. The occurrence of severe clinical symptoms in CHIKV infection (e.g., persistent arthralgia, destructive arthritis, and fulminant hepatitis) were documented by Renault et al. (*5*) during an outbreak in La Réunion Island during 2005–2006 (*2*). The severity of the outbreaks in the Indian Ocean was further correlated with the occurrence of specific mutations in the CHIKV genome that enabled highly efficient transmission of the mutated Indian Ocean lineage by *Ae. albopictus* mosquitoes (*2*,*4*). Later, chronic polyarthralgia and CHIKV infection–related deaths, most in the elderly and patients with co-morbid conditions, were reported in the Caribbean and the Pacific regions during outbreaks caused by the CHIKV Asian genotype (*4*). Zika-related neurologic disorders and a 20-fold increase in the incidence of Guillain-Barré syndrome were first reported dur-

ing the outbreak in French Polynesia during 2013–2014. Cases of Guillain-Barré syndrome were also recorded during the Zika outbreak in Brazil (*6*). Moreover, soon after health authorities in Brazil warned of an increase in the prevalence of microcephaly in newborns that might be associated with Zika virus infection in mothers during pregnancy, health authorities in French Polynesia confirmed that neurologic congenital abnormalities also had been observed during the Zika outbreak there (*6*).

Other lessons learned from the emergence of CHIKV and Zika virus in small tropical islands include evidence of non-vectorborne virus transmission and its associated public health implications. Perinatal transmission of Zika virus to a neonate was first described in infected pregnant women in French Polynesia, and possible transplacental transmission was further corroborated by the detection of the virus in amniotic fluid samples of 2 pregnant women in Brazil whose fetuses had been diagnosed with microcephaly (*6*). Sexual transmission of Zika virus, suggested by Foy et al. (*7*), was corroborated by detection of virus in the semen of a patient in French Polynesia (*8*). To prevent transmission of CHIKV and Zika virus by blood transfusion, local blood banks in French Polynesia and the Caribbean adjusted their algorithms for blood donation and screening of blood products during outbreaks (*9*,*10*).

When we observe the geographic distribution of DENV, CHIKV, and Zika virus over the past decade, DENV expansion appears to have been a continuous process. However, the emergence of CHIKV, first in the Indian Ocean and later in the Caribbean, and the emergence of Zika virus in the Pacific has dramatically expanded the reach of these viruses (Figure).

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Seroepidemiologic Screening for Zoonotic Viral Infections, Maputo, Mozambique

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To the Editor: In sub-Saharan Africa, febrile patients are often assumed to have, and are treated for, malaria, but when tested, many are malaria-negative. Because emerging diseases, such as chikungunya virus (CHIKV) and dengue virus (DENV) infections, cause outbreaks around the world (*1–3*), the importance of these pathogens has become more evident. However, low-income countries have limited epidemiologic data on alternative diagnoses to malaria (*4*,*5*) and poor laboratory capacity (*1*), which restrict further diagnostic investigations. An early study in Mozambique during the 1980s found antibodies to Rift Valley fever virus

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(RVFV) in 2% of pregnant women (*6*). More recently, a RVFV seroprevalence of 36.9% among cattle in the Maputo Province was shown in 2010–2011 (*7*). Furthermore, the movement of humans from rural areas to major cities, particularly to the capital of Maputo, might affect human illnesses and disease pattern of zoonotic viruses (*3*).

We conducted a pilot study on CHIKV, DENV, hantavirus, RVFV, and West Nile virus (WNV) epidemiology in Mozambique. Ethical approval (registration no. IRB00002657) was granted by the National Bioethics Committee in Mozambique and by the Regional Ethical Review Board at Karolinska Institutet, Stockholm, Sweden (permit no. 2012/974–31/3).

During 2012–2013, a total of 78 febrile patients were prospectively enrolled when they sought medical attention at the Polana Caniço Health Center and Mavalane Health Center (catchment area 4,663 km^2, estimated population 46,184 inhabitants) in the suburban area of Maputo city. All included patients answered a questionnaire and were initially screened for malaria by blood smear light microscopy; 15 were positive for malaria (Table). Patients' median age was 29 years (37 years for seropositive patients) and ranged from 5 to 78 years. Forty-six (59%) were female. Fifty-eight (74%) reported recent exposure to mosquitoes. None of these persons had a history of international travel, and none had received a yellow fever vaccination.

Sixty (77%) patients provided paired acute- and convalescent-phase blood samples, with a minimum of 14 days (median 33 days) between samples. Serum samples were sent to the Public Health Agency of Sweden and blindly screened at a titer of 1:20 for IgG to CHIKV, DENV, hantavirus, RVFV, and WNV by using in-house indirect immunofluorescence assays as described for DENV by Vene et al. (*8*). Screening for IgG were done on convalescent-phase serum samples or, when those was not available, on acute-phase serum samples. Further immunofluorescence analyses for titer increases were performed for patients for whom paired serum samples were available and screening results were positive for IgG; however, no titer increases were found. Serum from admittance were tested for DENV IgM and WNV IgM by using commercial assays according to manufacturers' instructions (Panbio Dengue IgM Capture ELISA E-DEN01M/E-DEN01M05, Standard Diagnostics, Inc., Yongin-si, South Korea; Serion ELISA classic ES-R14M West Nile Virus IgM, Institut Virion/Serion GmbH, Würzburg, Germany); 2 samples were positive for DENV IgM but none for WNV IgM. All acute serum samples were screened by using 1-step real-time reverse transcription PCR for CHIKV, RVFV, WNV (in-house validated assays), and DENV (*9*). Results were negative for viral RNA.

Twenty-three (29%) of the 78 patients had a positive serology result from acute- or convalescent-phase serum samples for ≥1 of the tested viral pathogens (Table). The main finding was CHIKV IgG in 15 (19%) patients. Ten (13%) patients had positive results for DENV, including 2 DENV IgM–positive samples.

The seroepidemiologic findings in this pilot study in Maputo strongly suggest possible and neglected alternative causes of febrile illness in Mozambique. Antibodies to CHIKV were found in 19% of the patients, which was a novel finding for Mozambique but corresponded well with other reports on the spread of CHIKV in tropical and subtropical areas of the world (*2*,*3*). DENV antibodies were present in 13% of the study population, representing a new finding in southern Mozambique; previous outbreaks have been reported from the northern part of the country (*5*). The median age of the seropositive patients (37 years) was higher than for the group as a whole (29 years), which might reflect increased exposure to zoonotic viruses over time. One patient was IgG positive for RVFV, a potentially emerging cause of fever in Mozambique, especially in view of recent reports of RVFV in cattle (*7*). The samples positive for both DENV and WNV IgG could represent previous independent infections with these viruses, co-infection, or cross-reactivity, which are common for flavivirus IgG (*10*).

Overall, results indicate that exposure to vectorborne viruses in persons living in suburban areas of Maputo city is frequent, suggesting that infections with CHIKV, DENV, and RVFV infection should be considered as alternative diagnoses for patients with febrile illness in these settings. On the basis of these results, more extensive research is planned on the epidemiology of zoonotic viral infections in Mozambique.

Table. Results of screening for viral antibodies and malaria parasites in 78 febrile patients, Maputo, Mozambique, 2012–2013*

Organism	No. (%) positive†
Chikungunya virus	15 (19.2)
Dengue virus	10 (12.8)‡
Hantavirus	0
Rift Valley fever virus	1 (1.3)
West Nile virus	3 (3.8)
Malaria parasites	15 (19.2)§

*Viral antibody–positive patients had positive IgG or IgM response for ≥1 of the zoonotic viruses in acute- or convalescent-phase serum samples. The overall malaria screening results for the study cohort is also presented
†Three of the 23 serology-positive patients were positive for dengue virus and West Nile virus IgG, of whom 2 were also positive for chikungunya virus IgG and 1 for Rift Valley fever virus IgG.
‡Including 2 dengue virus IgM-positive samples.
§Three of 15 malaria-positive patients had a positive serologic finding (2 for dengue virus IgG and 1 for chikungunya IgG).

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Hemorrhagic Diathesis in *Borrelia recurrentis* Infection Imported to Germany

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To the Editor: Relapsing fevers are paroxysmal bloodstream infections caused by spirochetes of the genus *Borrelia*. Louseborne relapsing fever (LBRF; i.e., epidemic relapsing fever) is caused by *B. recurrentis* and transmitted by the human body louse (*Pediculus humanus*). Soft ticks of the Argasidae family (e.g., *Ornithodorus moubata*) are vectors for tickborne relapsing fever (TBRF) borreliae, which encompass several human-pathogenic species. In Europe, LBRF was epidemic in the early 20th century but is now rarely seen. We report an infection with *B. recurrentis* imported to Germany by a Somalian refugee who had high fever and hemoptysis and describe the process of molecular diagnosis.

In August 2015, an 18-year-old man sought asylum in Germany after travel through Somalia, Ethiopia, Sudan, Libya, and Italy. He reported general weakness and fever while in Libya, ≈16 days before seeking care, and started coughing up blood after arriving in Italy. At hospital admission in Germany, he had a temperature up to 40.4°C, cough, and hemoptysis; his suspected diagnosis was tuberculosis. No ectoparasites were reported or found on physical examination. Abnormal laboratory findings included relative neutrophilia (91% [reference 39%–77%]), thrombocytopenia (platelets 112×10^3/mL [reference $160–385 \times 10^3$/mL]), and prolonged activated partial thromboplastin time (APTT) (Figure, panel A). Because of highly elevated levels of C-reactive protein (250 mg/L [reference <5 mg/L]) and procalcitonin (16.4 μg/L [reference <0.5 μg/L]), the patient was treated with ceftriaxone (2g/d intravenously), metronidazole (500 mg/d intravenously), and paracetamol (acetaminophen). Repeated examinations of Giemsa-stained thick and thin blood slides were negative for malaria parasites. Blood cultures, tests for tuberculosis, and PCRs for Rift Valley fever, yellow fever, dengue, and chikungunya viruses also were negative. With antimicrobial therapy, the patient's fever declined within 12 hours, but platelet counts further decreased and APTT continued to increase (Figure, panel A).

The patient's symptoms and travel history raised suspicion of a spirochete infection. A plasma sample from his second day in the hospital tested positive for *Borrelia* spp. 16S DNA by real-time PCR (*1*). Retrospective microscopy revealed a low number of extracellular spirochetes in thin blood smears (Figure, panel B). The antimicrobial regimen was changed to doxycycline (100 mg 2×/d) on day 7 after admission and, because species identification had not been completed, continued for 10 days. No signs of a Jarisch-Herxheimer reaction were seen. During days 4–9 after admission, APTT, platelet counts (Figure, panel A), and C-reactive protein values returned to normal, and the patient was discharged.

For species identification, we amplified the entire coding sequence of *glpQ* (glycerophosphodiester phosphodiesterase) with newly designed primers (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/5/15-1557-Techapp1.pdf). The amplicon was 100% (1,002/1,002 bp)

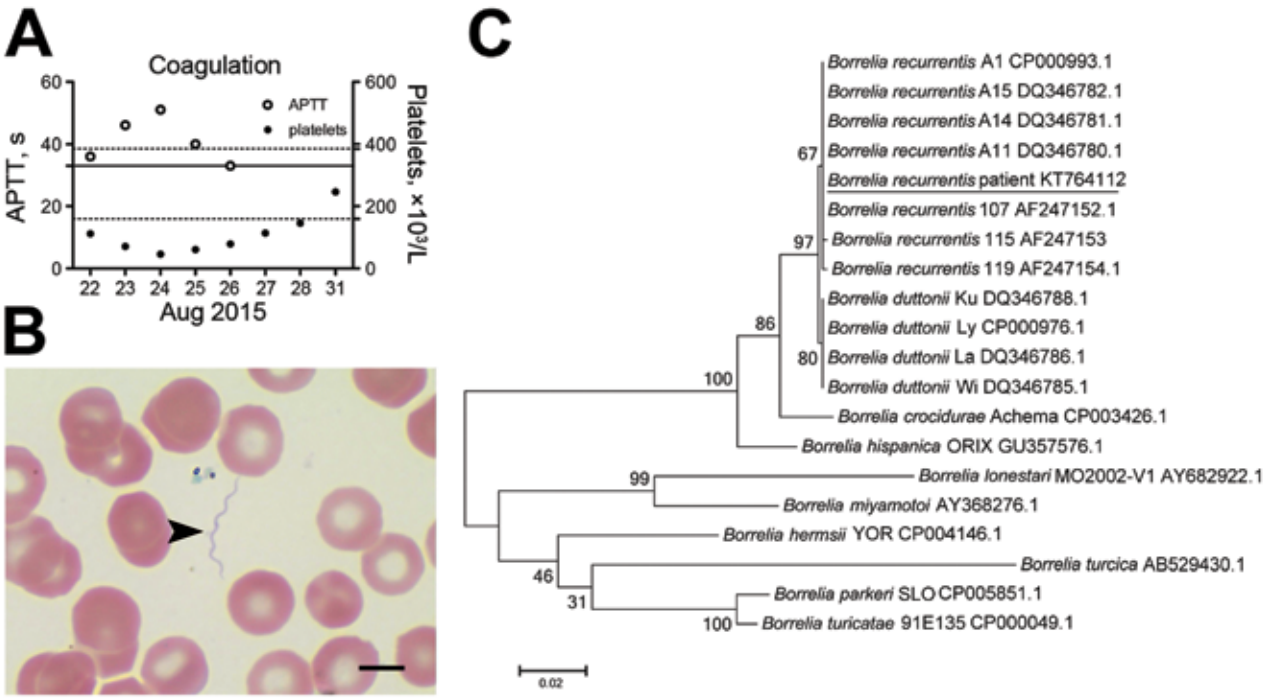

Figure. Laboratory findings of hemorrhagic diathesis in an 18-year-old Somalian refugee to Germany with *Borrelia recurrentis* infection, August 2015. A) Time course of coagulation parameters (thrombocytopenia and prolongation of activated partial thromboplastin time [APTT]). B) Extracellular spirochetes demonstrated by light microscopy (arrowhead). Representative image detail from thin blood smear, Giemsa stain. Scale bar indicates .5 mm. C) Molecular phylogenetic analysis of *B. recurrentis* detected in patient blood. Multiple alignment of complete *glpQ* sequence (1,002 bp) with published reference sequences was performed by using BioEdit 7.0.5.3 software (Ibis Biosciences, Carlsbad, CA, USA) and analyzed by MEGA6.06 (http://www.megasoftware.net). The evolutionary history was inferred by using maximum-likelihood method based on the Tamura-Nei model. Bootstrap values are shown at the node of branches (1,000 bootstrap replications). The complete *glpQ* sequence was deposited in GenBank under accession no. KT764112. Scale bar indicates nucleotide substitutions per site.

identical to *B. recurrentis* A1 (GenBank accession no. CP000993.1) and 99% identical (999/1,002 bp) to *B. duttonii* Ly (GenBank accession no. CP000976.1). A phylogenetic analysis that included 7 published *glpQ* sequences from *B. recurrentis* and 4 from *B. duttonii* suggested that the detected pathogen clustered with *B. recurrentis* and not *B. duttonii* (Figure, panel C).

Borreliae have been recognized as a frequent cause of febrile infections in West and East Africa (*2*). Data on the incidence in immigrants are not available, but the recent increase in asylum seekers from East Africa arriving in Central Europe has increased attention of *Borrelia* as a pathogen to be included in differential diagnoses of febrile infections (*3*,*4*). Because symptom onset in the patient we report occurred in Libya, he most likely acquired infection on the African continent, although local transmission in Europe can occur (*4*).

Blood slide examination, which would show spirochetes, is routinely requested to detect *Plasmodium* parasites, but its sensitivity in detecting borreliae is strikingly inferior to molecular tools (15%–56%, depending on laboratory conditions) (*1*). Pan-*Borrelia* real-time PCRs enable sensitive detection of DNA in blood samples, followed by sequencing (*1*) or confirmatory PCRs for relapsing fever *Borrelia*–specific genes (e.g., *glpQ*) (*5*,*6*). *B. recurrentis* is genetically highly similar to *B. duttonii,* suggesting it might be a degraded subset of its tickborne counterpart rather than a distinct species (*7*). Yet, phylogeny of whole *glpQ* sequences enables separation of *B. recurrentis* from *B. duttonii* on the basis of distinct single-nucleotide variations. Alternatively, differentiation can be achieved by phylogenetic analysis of concatenated partial 16s, *glpQ* and *flaB* (flagellin) sequences (*5*). Differentiation between TBRF and LBRF is crucial for the correct clinical decision on therapy duration, independent of the antimicrobial substance chosen: at least 7 days of treatment is recommended for TBRF to prevent relapses after early invasion of spirochetes into the central nervous system (*8*), whereas a single-dose regimen is sufficient for LBRF (*9*), although longer treatment courses tend to be used.

In summary, our report emphasizes that LBRF can be complicated by pulmonary hemorrhages associated with impaired platelet and plasmatic coagulation (*10*), which

can be mistaken for signs of tuberculosis. Considering the poor hygienic conditions among refugees, LBRF has become an important differential diagnosis in Europe in times of increasing migration.

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Crimean-Congo Hemorrhagic Fever Virus IgG in Goats, Bhutan

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To The Editor: Crimean-Congo hemorrhagic fever (CCHF) is a highly infectious tickborne disease caused by a high-risk group of viruses belonging to the family *Bunyaviridae* (*1,2*). In humans, the overall case-fatality rate of CCHF is ≈30%, but in severe and hospitalized patients, fatalities may be up to 80% (*3,4*). CCHF is widespread in various countries in Africa, Asia, and Europe; the virus had been identified in humans in China, Pakistan, and Afghanistan and has been recently reported for the first time in humans in India (*4–7*). Humans can be infected by bites from infected ticks, mainly of the *Hyalomma* genus; by unprotected contact with blood or tissue of viremic patients; or during slaughtering of infected animals. In addition, nosocomial infections are found in humans (*1,4,8,9*).

Fatal cases of CCHF in humans were confirmed in Ahmadabad in India in 2011, but a recent serosurvey in livestock showed that this disease has widespread seroprevalence in domestic animals across India (*7–10*). Bhutan shares a long, porous border with India, and animals and humans frequently cross the border. Comprehensive surveillance was needed to determine the presence of CCHF virus (CCHFV) in livestock in Bhutan and to assess risk for zoonotic infection in humans.

During October 2015, in collaboration with the National Centre for Animal Health Bhutan, we retrospectively tested serum samples collected during April–May 2015 from 81 goats and 92 cattle for CCHFV-specific IgG by using ELISA kits (Sheep/goat anti-CCHFV IgG ELISA kit and Cattle anti-CCHFV IgG ELISA kit; National Institute of Virology, Pune, India), as described (*10*). CCHFV IgG was detected in 31 (38.2%) goats; no cattle had positive results. The samples from goats, which were collected in early 2015 as part of surveillance of peste des petits ruminants, originated from the southern district of Sarpang, which shares a porous border with the state of Assam in India (Figure). The samples from cattle were collected

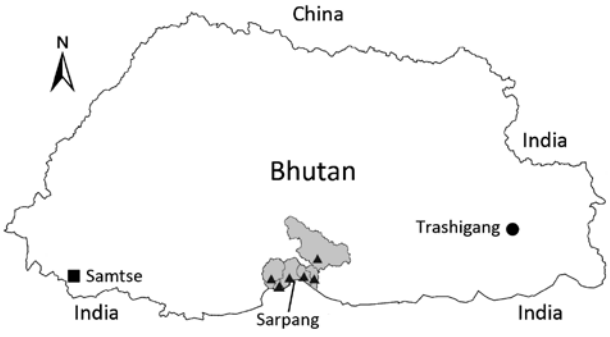

Figure. Locations in Bhutan where serum samples were collected from goats (triangles) and cattle (square and circle) and tested for Crimean-Congo hemorrhagic fever virus. The shaded area shows the boundaries of Sarpang district and subdistricts, where samples from goats were collected.

from the National Nublang Breeding Center (Trashigang district) and the National Jersey Breeding Center (Samtse district) (Figure). Findings indicated that all goats that tested positive for CCHFV were reported to have been either bred within households that kept goat herds or procured from other villages within the district. Exact sources of those seropositive goats could not be ascertained. However, in a few instances in the past, breeding goats (male and female) were procured from India by the Bhutan government and distributed to farmers for breed improvement. We also believe that cross-border movement of animals and unofficial imports of goats by farmers along the porous borders of southern Bhutan likely occurred. Furthermore, a large number of dairy cattle are imported annually from India for enhancing milk production and breeding purposes. Not all imported animals (both cattle and goats) were tested for CCHF because of a lack of diagnostic facilities and the negligible occurrence of the disease in livestock.

Our findings indicate that the risk of importing emerging infectious diseases along with live animals poses a serious risk to public health. Consequently, detailed risk-based surveillance is necessary to understand the complete scenario of CCHFV prevalence in livestock in Bhutan because *Hyalomma* tick species, the primary vectors of CCHF, are present on animals here. In addition, a survey among at-risk human populations is also needed. Findings from these surveillance activities would help institute more diagnostic facilities and risk-based surveillance and assist in developing a preparedness plan at the human–animal interface. Although our study has limitations because of the low number of serum samples tested from limited animal species from only 3 areas, the study provides evidence that CCHFV is circulating in goats in Bhutan.

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Community-Associated MRSA Infection in Remote Amazon Basin Area, Peru

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To the Editor: Two predominant community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) clones have been reported in South America: 1) sequence type 30 staphylococcal cassette chromosome *mec* IV (ST30-SCC*mec* IV) (USA 1100), first found in Uruguay (2002) and later in Brazil and Argentina (2005); and 2) ST8-SCC*mec* IVc/E (USA300–Latin American variant), found predominantly in Ecuador and Colombia (2006–2008) (*1*). In hospitals in Colombia, USA300–Latin American variant has replaced the most common hospital-associated lineage, known as the Cordobes/Chilean clone (MRSA ST5-SCC*mec* I) (*2*). In Peru, a limited number of imported cases of CA-MRSA have been reported (*3*). We describe a case of CA-MRSA infection in a patient living in a remote area of the Amazon Basin of Peru.

The patient was a 58-year-old woman who was hospitalized in June 2014 for a skin ulcer. She had been well until 10 days before admission, when she noticed a papule on her right arm, followed the next day by localized swelling and redness. Three days later, spontaneous secretion of a purulent material was noted. At the time of admission, the patient had no fever or constitutional symptoms; the ulcer was deep with irregular borders (≈10 × 4 cm) and active purulent secretion (Figure, panel A). Other physical examination findings were unremarkable.

For the past 2 years, the patient had lived in a remote, rural, jungle village in Peru. She was a housewife but also farmed in nearby areas. There were ducks, chickens, and guinea pigs on the farm where she lived. Her village had neither running water nor roads and almost no access to healthcare (reaching the nearest healthcare center required a 36-hour boat trip). She previously experienced several episodes of malaria (most recently in February 2014), for which she received antimalarial medication provided by a Brazilian military post at the border of Peru. She had never taken antimicrobial drugs and had not traveled in the past 2 years. She first noticed the skin lesion on the first day of an 8-day boat trip from her home village to Iquitos, the largest city in the Peruvian Amazon Basin.

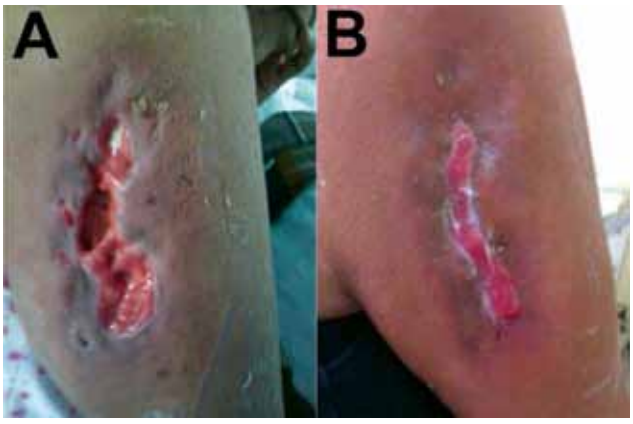

Figure. A) Untreated community-associated methicillin-resistant *Staphylococcus aureus* ulcer on the right arm of a 58-year old woman from a rural area of the Amazon Basin, Peru. B) The same ulcer after 19 days of treatment with vancomycin and trimethoprim/sulfamethoxazole.

Cultures from wound exudate and skin biopsy samples yielded *S. aureus* resistant to oxacillin, tetracycline, and erythromycin and susceptible to ciprofloxacin, gentamicin, rifampin, and trimethoprim/sulfamethoxazole (susceptibility testing performed by an agar dilution method). D test showed inducible resistance to clindamycin. The presence of *mecA* and the genes (*lukS-PV*, *lukF-PV*) encoding Panton-Valentine leucocidin (PVL) were confirmed by PCR.

The isolate was characterized as MRSA ST6-t701-SCC*mec*V. Whole-genome sequence analyses identified a predicted protein with 100% aa identity (98% coverage) to the truncated b-hemolysin of the reference genome of *S. aureus* USA300_FPR3757 (GenBank accession no. gb|ABD20946.1|) and the prophage groups 1, 2, and 3. Pulsed-field gel electrophoresis (PFGE) exhibited a pulsotype different from other typical CA-MRSA PFGE patterns found in MRSA from Latin America, labeled as CA-MRSA 120 (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/5/15-1881-Techapp1.pdf). Intravenous clindamycin (600 mg every 8 hours for 5 days) was empirically prescribed, after which treatment was switched to vancomycin (1 g every 12 hours for 1 week). Subsequently, the patient received trimethoprim/sulfamethoxazole (160/800 mg every 12 hours for 1 week). The clinical evolution was satisfactory, and the infection resolved (Figure, panel B).

This case of a skin and soft tissue infection caused by a CA-MRSA ST6-t701-SCC*mec* V PVL-producing organism is notable for several reasons. First, infection occurred in a remote rural jungle area of Peru at the border with Brazil

and Colombia and resembles the first cases of CA-MRSA described in the early 1990s as occurring in indigenous people living in remote areas of Western Australia (*4*). Second, considering that the most predominant CA-MRSA clones in Latin America carry SCC*mec* IV (*1*,*5*), finding SCC*mec* V in this isolate was not expected. MRSA carrying SCC*mec* V have been well characterized as colonizers and agents of infection in animals and in humans in close contact with animals (mainly in Europe but also in other parts of the world) (*6*). These livestock-associated MRSA clones predominantly belong to ST97 (which are usually not PVL producers) and ST398. In addition, ST398 SCC*mec* V MRSA isolates from pigs in Peru have been described (*7*). Of note, methicillin-susceptible *S. aureus* t701 and MRSA t701 carrying SCC*mec* II have recently been found in China, isolated from patients during food poisoning outbreaks and from colonized pork butchers, respectively (*8*,*9*). In South America, isolation of non–PVL-producing MRSA t701 (carrying SCC*mec* IVc) and methicillin-sensitive S. *aureus* t701 from colonized inpatients has been well described (*10*). Although speculation that animal carriage might have played a role in this infection is tempting, further studies are needed to recognize the origin of this MRSA ST6-SCC*mec* V PVL producer in this area of the Amazon Basin.

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Exposure to Bat-Associated *Bartonella* spp. among Humans and Other Animals, Ghana

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To the Editor: Human contact with wildlife is a leading cause of disease spillover. Bats, in particular, host numerous zoonotic pathogens, from henipaviruses to lyssaviruses (*1*). In Ghana, the straw-colored fruit bat (*Eidolon helvum*) frequently and closely interacts with humans through roosting in urban areas and human harvesting of bushmeat. Large colonies live in Accra, the capital city, and >128,000 bats, on average, are hunted for food yearly in southern Ghana alone (*2*). Serologic evidence of human infection with novel paramyxoviruses from *E. helvum* bats (*3*) supports concerns regarding this contact. In addition, Kosoy et al. (*4*) isolated several new strains of *Bartonella* that were found in >30% of *E. helvum* bats, whereas Billeter et al. found *Bartonella* in 66% of their ectoparasites (*5*), with *Bartonella* transmissibility to other species unknown. This prevalence causes concern because many *Bartonella*

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species are zoonotic and cause substantial human disease (*6*). Previous studies of febrile patients in Thailand have shown prevalence rates of ≤25% for antibodies against zoonotic *Bartonella* species (*7*). Serologic studies have been conducted in Europe and in the United States, but few studies have examined such prevalence in Africa among patients and in the general population (*8*).

To address these concerns, we conducted a prevalence study in Ghana, West Africa, for evidence of bat-associated *Bartonella* infection in humans and other common animal species. We sampled humans who had close contact with fruit bats and also sampled domestic animals that lived around the bat colonies.

We obtained serum samples from 335 volunteers from Accra and the Volta region who had close contact with *E. helvum* bats and also sampled 70 domestic animals that lived underneath bat colonies (5 cats, 23 chickens, 7 cows, 6 dogs, 21 goats, 8 sheep) in Accra. We used 3 testing approaches: culture, PCR, and indirect immunofluorescent assays for serologic testing. We tested serum specimens for antibodies against *B. henselae*, *B. quintana*, *B. clarridgeiae*, *B. vinsonii vinsonii*, *B. elizabethae*, and *Bartonella* strain E1–105, which had been isolated from *E. helvum* bats (*6*).

All culture results for human and domestic animal samples were negative for *Bartonella* species. One human serum sample was positive for *B. clarridgeiae* by PCR, which was confirmed by repeat testing. No other human samples were consistently positive by PCR. Of 70 animal blood clots and 62 serum samples tested by using PCR, 1 serum sample from a cat tested positive for *B. henselae*. One human serum sample was positive by immunofluorescent assay for antibodies against *B. henselae* at titers of 1:128, another had reactivity to *B. henselae* at 1:64, and 1 sample was reactive at 1:32. Five human serum samples were reactive to *B. quintana* at titers of 1:32.

The absence of evidence of any human exposure to bat-associated *Bartonella* suggests that the species isolated from *E. helvum* bats never or rarely infects humans in Ghana. If *Nycteribiidae* bat flies serve as the vector for *Bartonella* transmission between bats as hypothesized, then the high host specificity of these vectors (*8*) could explain why little infection is spilling over to other species. However, no experimental studies have confirmed that bat flies are competent vectors of bat-associated *Bartonella* species or that these ectoparasites only bite bats. These facts must be confirmed because bat flies are occasionally found on other animals and whether the parasites can successfully use these animals as hosts is unknown (*9*). Although further studies are needed to clarify the dynamics of *Bartonella* species infection in *E. helvum* bats, as well as the species' zoonotic potential, the current risk of spillover of this bat-associated *Bartonella* species appears low in West Africa. This fact may be useful in directing limited public health resources.

The seroprevalence to *B. henselae* in healthy human participants in this study was <1%. The low levels of seropositivity to *B. henselae* and *B. quintana* are consistent with those found in the only other study on *Bartonella* in humans in sub-Saharan Africa: a survey of 155 subjects in the Democratic Republic of Congo showed 1% seroprevalence of *B. henselae* and <1% seroprevalence of *B. quintana* (*8*).

The results of study in the Democratic Republic of Congo and this study contrast with some studies in Asia and Europe, which show higher rates of human exposure to *Bartonella* species. For example, a study of febrile patients in Thailand found serologic evidence of exposure to *Bartonella* infection in 25% of patients (*7*).

Laudisoit et al. (*8*) were, to our knowledge, the first to report evidence of *Bartonella* infection in humans in Africa. Our study contributes to this nascent effort of understanding *Bartonella* on the continent. Because a substantial proportion of *Bartonella* prevalence studies have been done on hospital patients, our study provides a survey of the general population to help determine background infection rates and illuminate the complex risks posed by this zoonosis.

The research for this article was carried out during fieldwork in southern Ghana; at the 37 Military Hospital in Accra; at the University of Cambridge, Cambridge, UK; and at the Centers for Disease Control and Prevention, Fort Collins, Colorado, USA.

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Isolation of Zika Virus from Febrile Patient, Indonesia

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To the Editor: Arthropodborne viruses (arboviruses) cause substantial human disease worldwide and have a pronounced effect on public health throughout Asia. Zika virus, discovered in Uganda in 1947 (*1*), is a flavivirus related to the following viruses: dengue (DENV), West Nile, Japanese encephalitis, and yellow fever. Like DENV, Zika virus is transmitted by *Aedes* mosquitoes. Zika virus emerged as a public health problem in 2007, when it caused an epidemic in Micronesia (*2*). Since then, the virus has caused epidemics elsewhere in the Pacific islands (*3*) and recently emerged in South America (*4*). Zika virus has been reported to cause mild and self-limited infection that can be misdiagnosed as dengue because of similar clinical features and serologic cross-reactivity (*2*). Zika virus has not, however, been reported to cause substantial thrombocytopenia or result in the serious vascular leakage that can be fatal in DENV infection.

Until recently, most evidence for Zika virus infection in Asia, including in Indonesia (*5*), has been serologic, but recent virus strains isolated from persons in Thailand (*6*), the Philippines (*7*), and Cambodia (*8*) have begun to clarify its genomic diversity. Phylogenetically, Zika virus appears to have 2 major lineages, African and Asian (*9*).

During December 2014–April 2015, a confirmed outbreak of dengue (determined by reverse transcription PCR [RT-PCR] for DENV and nonstructural protein 1 [NS1] antigen detection; data not shown) occurred in Jambi Province, central Sumatra, Indonesia. We received samples from 103 case-patients with clinically diagnosed dengue; these samples had been negative for DENV by RT-PCR, NS1 antigen detection, or evidence of seroconversion by ELISA (data not shown). We tested the samples for other viruses using alphavirus and flavivirus RT-PCR (targeting genome positions 6533–6999 and 8993–9258, respectively). In parallel, we attempted virus isolation using Vero cells.

One sample, JMB-185, came from a 27-year-old man who sought treatment at the Jambi city hospital 2 days after illness onset with a sudden high fever, headache, elbow and knee arthralgia, myalgia, and malaise. He did not exhibit some common clinical characteristics of Zika virus infection (*10*), including maculopapular rash, conjunctivitis, or peripheral edema. Hematologic testing revealed lymphocytopenia and monocytosis; platelet count was within reference range. Results of all assays were negative for DENV, including NS1 antigen detection with NS1 Ag Rapid Test (SD Bioline, Kyong, South Korea); PanBio Dengue Early NS1 ELISA (Alere, Brisbane, Australia); PanBio Dengue Duo IgM and IgG ELISA (Alere); and Simplexa real-time RT-PCR (Focus Diagnostics, Cypress, CA, USA). The illness was self-limiting, and the patient recovered 2 days after he sought treatment without any complications.

Of the 103 DENV-negative specimens we tested, only JMB-185 was positive for flavivirus and displayed cytopathic effects when cultured in Vero cells for 10 days. A subsequent passage was performed, and supernatants from both passages were tested for flaviviruses by RT-PCR. A 265-bp amplicon was generated from JMB-185 by using flavivirus consensus primers. This consensus amplicon product had ≈85% nucleotide identity with the prototype Zika virus (strain MR 766, 1947, Uganda). An additional larger amplicon was generated (nt 9278–9808 of NS5 gene), and a phylogenetic tree was constructed based on the partial sequence of the NS5 region (530 bp) for JMB-185 (GenBank accession no. KU179098) and other Zika virus sequences, including those from Cambodia, Yap Island, Thailand, and the Philippines (Figure). Phylogenetic analysis indicated that JMB-185 belonged to the Zika virus Asian lineage and had 99.24% nucleotide identity to an isolate from a Canadian visitor to Thailand (*10*). It was also close to a Zika virus strain isolated from an Australian traveler who had visited Java (on the basis of a different NS5 region; data not shown). The original serum and passage samples were

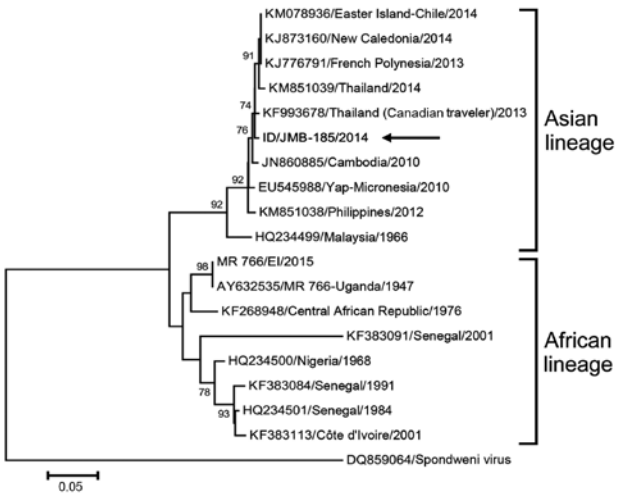

Figure. Phylogenetic tree comparing Zika virus isolate from a patient in Indonesia (ID/JMB-185/2014; arrow) to reference strains from GenBank (accession numbers indicated). The tree was constructed from nucleic acid sequences of 530 bp from the nonstructural protein 5 region by using the minimum evolution algorithm in MEGA 6 (http://www.megasoftware.net). Numbers to the left of the nodes are bootstrap percentages (2,000 replications). Bootstrap values <70 are not shown. The tree was rooted with the Spondweni virus isolated in South Africa as the outgroup. Scale bar indicates nucleotide substitutions per site.

further tested with Zika virus–specific real-time quantitative RT-PCR (*2*) by using the QuantiTect Probe RT-PCR Kit (QIAGEN, Valencia, CA, USA) with amplification in the iCycler iQ5 (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions. Viral titers of JMB-185, as determined by real-time quantitative RT-PCR, were 4.25×10^3 PFU, 5.07×10^7 PFU, and 7.33×10^6 PFU for the clinical sample, first passage, and second passage, respectively.

The isolation and characterization of Zika virus from a resident with no travel history confirm that the virus is circulating in Indonesia and that, by mimicking mild dengue infection, this infection is likely contributing to the large number of undiagnosed cases of acute febrile illness. Although reported human cases of Zika virus infection have been rare in Southeast Asia (*1*), confusion with dengue and difficulty in obtaining a laboratory diagnosis are likely causing its incidence to be underestimated. Surveillance must be implemented to evaluate and monitor the distribution of Zika virus and the potential public health problems it may cause in Indonesia.

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Fatal Sickle Cell Disease and Zika Virus Infection in Girl from Colombia

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To the Editor: Zika virus, a mosquito-borne flavivirus, causes a usually self-limiting febrile and exanthematic arthralgia syndrome that resembles dengue and chikungunya (*1*). This arboviral disease has emerged in tropical areas of Latin America, particularly in Brazil and Colombia (*2*), as a public health threat in 2015 and has spread into areas to which dengue virus (DENV) and chikungunya virus (CHIKV) are endemic (*1–4*).

Cases of severe and fatal Zika virus infection have not been described (5), and the spectrum of clinical disease remains uncertain in the setting of rapidly evolving epidemics of this arbovirus in Latin America (1). We report a person with sickle cell disease who acquired a Zika virus infection and died.

The patient was a 15-year-old girl who in October 2015 came to the outpatient clinic of the Hospital of Malambo (a primary-level public hospital) in Malambo (Atlántico Department) in northern Colombia. In this region, during September 22, 2015–January 2, 2016, a total of 468 suspected cases of Zika virus infection and 4 reverse transcription PCR (RT-PCR)–confirmed cases have been reported. This patient had a high fever (temperature >40°C), arthralgias, retro-ocular pain, abdominal pain, myalgias, and jaundice for the previous 4 days. She had sickle cell disease for 5 years (hemoglobin genotype SC identified by DNA analysis), but no previous hospitalizations or episodes of vaso-occlusive crises. She had never had dengue, chikungunya, or acute chest syndrome.

At admission to the hospital, the patient had a pulse rate of 112 beats/min, a respiratory rate of 24 breaths/min, a blood pressure of 110/70 mm Hg, and a temperature of 39.0°C. She had abdominal pain, no petechiae, and no lymphadenopathy. The patient was given acetaminophen. Results of a neurologic assessment were unremarkable. Clinical laboratory findings are shown in the Table.

Given these manifestations, she was given a diagnosis of a DENV infection and referred to Barranquilla Hospital Metropolitano (Barranquilla, Colombia) where she was admitted 1 day later. Physical examination showed a pulse rate of 122 beats/min, a respiratory rate of 34/min (peripheral capillary oxygen saturation 93%), a blood pressure of 112/58 mm, and a temperature of 37.5°C. She had generalized jaundice, respiratory distress, severe abdominal pain, hepatomegaly, and splenomegaly, but no lymphadenopathy. The patient was conscious (stuporous) and had a Glasgow Coma Scale score of 13. Cardiovascular assessment showed

Table. Clinical laboratory results for 15-year-old girl with sickle cell disease who died of Zika virus infection, Colombia*

Laboratory test	Baseline value	Value at hospitalization (Malambo)	Value 24 h later (ICU, Barranquilla)
Leukocyte count, × 10^9 cells/L	10.00	8.23	ND
Hemoglobin level, g/dL	7.00	8.10	4.20
Hematocrit, %	28.00	25.00	13.00
MCV, fL/erythrocyte	73.00	73.00	ND
Reticulocytes, %	1.00	1.00	ND
Total bilirubin, mg/dL	ND	2.97	ND
Direct bilirubin, mg/dL	ND	1.67	ND
Platelet count/mL	ND	54,000.00	76,000.00
PT, s	ND	ND	33.3 (control 13.10)
aPTT, s	ND	ND	45.0 (control 29.80)
ALT, mg/dL	ND	ND	2,245.00
AST, mg/dL	ND	ND	3,215.00
LDH , IU/L	ND	ND	441.00
Alkaline hemoglobin electrophoresis, %			
HbS	ND	ND	62.50
HbC–E	ND	ND	37.50
HbF	ND	ND	0.00
Malaria thick and thin blood smears†	Not done	Not done	–
HIV-1 and HIV-2 ELISA†	Not done	Not done	–
MAT for *Leptospira* spp.†	Not done	Not done	–
RT-PCR for DENV†	Not done	Not done	–
RT-PCR for CHIKV†	Not done	Not done	–
RT-PCR for YFV†	Not done	Not done	–
RT-PCR for ZIKV†	Not done	Not done	+

*ICU, intensive care unit; ND, not determined; MCV, mean corpuscular volume; PT, prothrombin time; aPTT, activated partial thromboplastin time; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; Hb, hemoglobin; –, negative; MAT; microscopic agglutination test; RT-PCR, reverse transcription PCR; DENV, dengue virus; CHIKV, chikungunya virus; YFV, yellow fever virus; ZIKV, Zika virus; +, positive.
†Blood samples were obtained 5 days after illness onset. These tests were performed at the National Reference Laboratory of the National Institute of Health, Bogotá, Colombia.

tachycardia and a holosystolic murmur (grade II) but no other findings.

The patient was then transferred to the pediatric intensive care unit, where she was intubated and mechanical ventilation was initiated. Her condition was considered life threatening; the patient had severe acute respiratory distress syndrome and progressive hypoxemia despite ventilator treatment, and laboratory findings worsened (Table).

The patient was given transfusions of blood products for treatment of anemia and thrombocytopenia. Chest radiograph and ultrasound showed an extensive right-side hemothorax. The result of a Zika virus–specific real-time RT-PCR was positive (Table). Her clinical condition deteriorated. Despite intensive treatment, the patient did not recover and died 37 hours later. An autopsy showed hepatic panacinar necrosis, erythrophagocytosis of Kupffer cells, and severe decrease of splenic lymphoid tissue (functional asplenia) with multiple drepanocytes and splenic sequestration, but no signs of yellow fever or malaria (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/22/5/15-1934-Techapp1.pdf).

Although sickle cell disorders are not common in Colombia, their frequency is higher along the Caribbean coast (including Atlántico Department) and 2 times that of the rest of Colombia) (*6*). Although chronic diseases, such as sickle cell disorders, are considered to be a risk factor for development of severe dengue and chikungunya (*7*,*8*), no cases have been reported in association with Zika. Reports of patients co-infected with DENV and CHIKV are rare, few details are available, and mostly restricted to few fatal cases of dengue (*9*). In patients with dengue, deaths might be higher among those who have a hemoglobin SC genotype, as recently reported (*10*). Onset of vaso-occlusion in persons with sickle cell disorders is often triggered by inflammation, as has been reported in DENV infections and which probably occurred in our patient (*8*). This complication and severe splenic sequestration, detected by autopsy, probably caused her death.

In summary, this case indicates that patients with sickle cell disorders and suspected arboviral infections should be closely monitored. Given current epidemics of Zika virus infection in Colombia (746 RT-PCR–confirmed cases and 11,712 suspected cases during September 22, 2015–January 2, 2016), atypical and severe manifestations and concurrent conditions in patients should be assessed to prevent additional deaths (2).

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Detection of Autochthonous Zika Virus Transmission in Sincelejo, Colombia

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To the Editor: Zika virus is an arthropodborne member of the genus Flavivirus of the Spondweni serocomplex and is transmitted by Aedes mosquitoes (primarily Ae. aegypti in urban and periurban cycles). Zika virus emerged in Africa and has caused outbreaks of febrile disease that clinically resemble dengue fever and other arboviral diseases (1) but has been linked to neurologic syndromes and congenital malformation (2). Outbreaks have been reported in the Yap islands of the Federated States of Micronesia (3), French Polynesia (4), and Oceania; Brazil is currently experiencing the first reported local transmission of Zika virus in the Americas (5).

The future spread of Zika virus is unpredictable, but the history of the virus has been reminiscent of chikungunya virus (CHIKV), which reemerged in Africa and now circulates on all inhabited continents and is a major global health problem. Zika virus has been found in Colombia and is likely following the path of CHIKV, which reached the country in August 2014 (*6*). The virus co-circulates with other *Ae. aegypti*–transmitted arboviruses, including dengue virus (DENV) and yellow fever virus. We report Zika virus infection in Colombia and a recent ongoing outbreak in Sincelejo, Colombia, with resulting illness characterized by maculopapular rash, fever, myalgia/arthralgia, and conjunctivitis.

During October–November 2015, a total of 22 patients received a presumptive diagnosis of an acute viral illness by emergency department physicians at the Centro de Diagnostico Medico-Universidad de Sucre in Sincelejo. The patients began treatment for a dengue-like illness, and blood samples were obtained for diagnosis. The samples were analyzed at the Universidad de Sucre by using reverse transcription PCR (RT-PCR) to detect DENV, CHIKV, or Zika virus. Viral RNA was extracted from the serum samples by using the ZR Viral RNA Kit (Zymo Research, Irvine, CA, USA); reverse transcription was performed by using the Protoscript First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA). Amplicons were produced by using the OneTaq Quick-Load 2X Master Mix (New England Biolabs) with primers specific to DENV (*7*), CHIKV (forward: 5′-CGCCAACATTCTGCTTACAC-3′; reverse: 5′-AGGATGCCGGTCATTTGAT-3′), and Zika virus. The CHIKV amplification target was 649 bp of nonstructural protein 1 (NS1). A positive PCR for a partial region of the envelope (E) gene with primers ZIKVENVF and ZIKVENVR (*8*) was considered indicative of Zika virus infection. Zika virus primers specific for the E gene and NS1 were designed and used to amplify the E gene and NS1 for phylogenetic analyses, and amplicons were produced by using the OneTaq One-Step

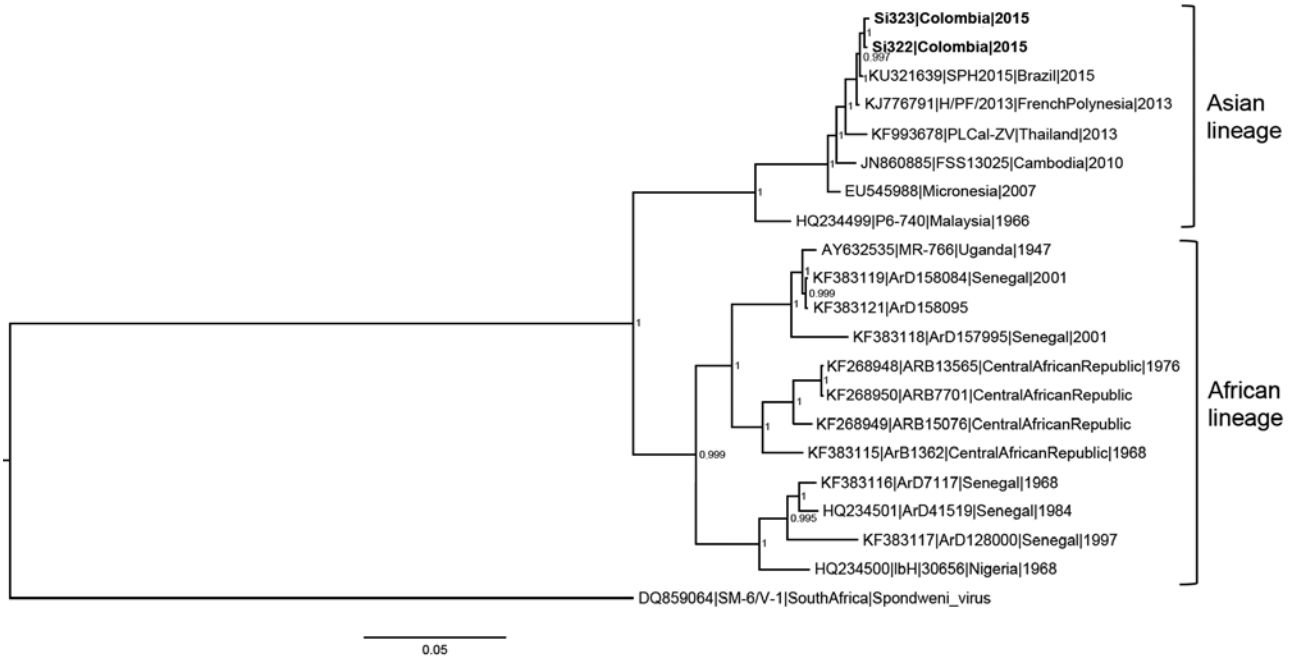

Figure. Majority-rule consensus tree based on Zika virus envelope and nonstructural protein 1 gene sequences (2,604 nt) of isolates from patients in Sincelejo, Colombia, October–November 2015, compared with reference isolates. The tree was constructed on the basis of Bayesian phylogenetic analysis with 8 million generations and a general time-reversible substitution model using MrBayes software version 3.2 (http://mrbayes.sourceforge.net). Numbers to the right of nodes represent posterior probabilities for corresponding clades. Samples sequenced in this study are in bold, and sequences are listed with GenBank accession numbers and are coded as accession no./strain/country/year of isolation when all information was available. The Colombia sequences are grouped with the Asian lineage of Zika virus. The tree was rooted with the Spondweni virus isolated in South Africa as the outgroup. Scale bar indicates nucleotide substitutions per site.

RT-PCR Kit (New England Biolabs). E gene and NS1 PCR products were sequenced at the University of Wisconsin–Madison Biotechnology Center (Madison, WI, USA).

Samples from all patients were negative by RT-PCR for DENV and CHIKV; samples from 9 (41%) patients were positive for Zika virus. Among those 9 patients, 7 (78%) were male; median age was 23; and none had a history of international travel. Zika virus was analyzed by sequencing the E gene and NS1 of 2 isolates. Phylogenetic analyses rooted with Spondweni virus showed that the Zika virus sequences (GenBank accession nos. KU646827 and KU646828) belonged to the Asian lineage (Figure) and were closely related to strains isolated during the 2015 outbreak in Brazil (*5*). The sequences also showed 99% identity with sequences from a Zika virus isolate from French Polynesia (GenBank accession no. KJ776791) (*9*). These data suggest that Zika virus circulating in Colombia could have been imported from Brazil, most likely as a result of tourism activities on Colombia's northern coast, where the first reported case was identified (the state of Bolivar).

We report Zika virus infection in Colombia in association with an ongoing outbreak of acute maculoexantematic illness. Since detection of Zika virus in Sincelejo, a total of 13,500 cases have been identified in 28 of the country's 32 territorial entities (*10*), all of which have abundant populations of *Ae. aegypti* mosquitoes and co-circulation of DENV and CHIKV. These circumstances highlight the need for accurate laboratory diagnostics and suggest that monitoring whether the virus spreads into neighboring countries (e.g., Ecuador, Peru, Venezuela, and Panama) is imperative.

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Health Precautions Taken by Travelers to Countries with Ebola Virus Disease

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To the Editor: To facilitate early recognition of Ebola virus disease (EVD), the New York City Department of Health and Mental Hygiene (DOHMH) actively monitored persons who had recently traveled from an EVD-affected country (*1*,*2*). Clinical manifestations of EVD are nonspecific and can resemble common travel-associated illnesses, such as malaria and influenza, both of which are potentially preventable through use of certain health precautions (*3*,*4*). Given the consequences of missing an EVD diagnosis, symptomatic persons under active monitoring who actually have non-EVD illnesses are often first isolated and tested for Ebola virus, which can delay appropriate care for the true cause of their illness and consume substantial resources. We evaluated the health precautions taken by persons traveling to EVD-affected countries.

During March 16, 2015–December 29, 2015 (the last day of EVD active monitoring by DOHMH), persons who underwent active EVD monitoring by DOHMH and who reported living in the United States for most of the previous

year were asked about health precautions taken when traveling to an EVD-affected country, regardless of whether they had symptoms. Health precautions assessed were whether a healthcare provider was visited for pretravel medical advice, whether malaria prophylaxis was used during the previous 7 days (if the date of departure from the EVD-affected country was within the previous 7 days), and whether influenza vaccination was received within the past year. Health precautions were examined by country visited, sex, age, reason for travel, and citizenship. Relative risks (RRs) and 95% CIs were calculated.

During the evaluation period, DOHMH actively monitored 4,230 persons, of whom 2,032 (48.0%) reported living in the United States. Among these 2,032 persons, only 1,265 (62.3%) received pretravel medical advice and 1,198 (59.0%) received influenza vaccination. Among the 1,992 persons whose date of departure from the EVD-affected country was within the previous 7 days of the date of data collection, 822 (41.3%) used malaria prophylaxis (Table).

The most common reason for travel to an EVD-affected country was to visit friends or relatives, which was reported by 1,655 (81.4%) of 2,032 persons. Female travelers were more likely than male travelers to use each of the health precautions. Persons who traveled for business reasons (RR 1.54, 95% CI 1.37–1.75) or for service-related reasons (humanitarian aid, missionary, volunteer, research, or military reasons; RR 2.07, 95% CI 1.78–2.40) were more likely to use malaria prophylaxis than those who traveled to visit friends or relatives, although there were no differences for receiving pretravel medical advice. US citizens were more likely to receive pretravel medical advice than citizens of the 3 EVD-affected countries and more likely to use malaria prophylaxis than citizens of Guinea (RR 0.76, 95% CI 0.65–0.89) or Sierra Leone (RR 0.65, 95% CI 0.48–0.88).

In summary, persons traveling to EVD-affected countries frequently did not use major health precautions, despite federal travel warnings for EVD-affected countries and the consequences of a febrile illness developing (*5*). Our findings are notable because New York City represents >20% of all persons actively monitored for EVD in the United States (more than any other jurisdiction) (*1*). Most persons reported in this study traveled to visit friends or relatives and were less likely to use malaria prophylaxis than those who traveled for business or

Table. Health precautions taken by 2,032 travelers to countries with Ebola virus disease who underwent active monitoring by the New York City Department of Health and Mental Hygiene after returning to the United States, March 16–December 29,2015*

	Health precaution†					
	Pretravel medical advice		Malaria prophylaxis‡		Influenza vaccine in past 12 mo	
Characteristic	No. (%)	RR (95% CI)	No. (%)	RR (95% CI)	No. (%)	RR (95% CI)
Country visited						
Guinea	960 (62.3)	0.91 (0.73–1.13)	**567 (37.4)**	**0.51 (0.41–0.62)**	932 (60.5)	0.89 (0.72–1.11)
Liberia	85 (57.8)	0.84 (0.65–1.09)	**71 (50.0)**	**0.67 (0.52–0.87)**	79 (53.7)	0.79 (0.61–1.02)
Sierra Leone	194 (63.4)	0.93 (0.74–1.17)	**158 (53.0)**	**0.71 (0.57–0.89)**	**161 (52.6)**	**0.77 (0.61–0.98)**
Multiple countries	26 (68.4)	Reference	26 (74.3)	Reference	26 (68.4)	Reference
Sex						
F	**574 (71.5)**	**1.27 (1.19–1.35)**	**409 (52.3)**	**1.55 (1.40–1.71)**	**561 (69.9)**	**1.35 (1.26–1.44)**
M	691 (56.2)	Reference	413 (34.1)	Reference	637 (51.8)	Reference
Age, y						
<5	**106 (82.2)**	**1.37 (1.24–1.51)**	**74 (57.4)**	**1.54 (1.30–1.83)**	**100 (77.5)**	**1.44 (1.29–1.61)**
5–14	**144 (80.5)**	**1.34 (1.22–1.47)**	**103 (57.9)**	**1.59 (1.36–1.84)**	**127 (71.0)**	**1.34 (1.20–1.50)**
15–24	82 (60.7)	1.01 (0.87–1.17)	58 (43.6)	1.16 (0.94–1.43)	75 (55.6)	1.08 (0.92–1.27)
25–44	509 (59.9)	Reference	312 (37.6)	Reference	454 (53.4)	Reference
45–64	384 (56.3)	0.94 (0.86–1.02)	254 (38.0)	1.01 (0.88–1.15)	**404 (59.2)**	**1.11 (1.02–1.21)**
≥65	40 (70.2)	1.17 (0.98–1.39)	21 (38.9)	1.05 (0.74–1.48)	**38 (66.7)**	**1.24 (1.02–1.51)**
Reason for travel						
Business	161 (61.9)	1.00 (0.91–1.11)	**140 (58.1)**	**1.54 (1.37–1.75)**	**135 (51.9)**	**0.86 (0.76–0.97)**
Education	12 (70.6)	1.13 (0.83–1.54)	7 (41.2)	1.09 (0.61–1.93)	7 (41.2)	0.68 (0.38–1.19)
Service-related§	45 (70.3)	1.13 (0.96–1.33)	**46 (78.0)**	**2.07 (1.78–2.40)**	37 (57.8)	0.96 (0.78–1.19)
Tourism	6 (33.3)	0.53 (0.28–1.03)	10 (55.6)	1.47 (0.97–2.24)	9 (50.0)	0.82 (0.52–1.30)
Visiting friends/relatives	1,030 (62.2)	Reference	613 (37.4)	Reference	1,001 (60.5)	Reference
Refused/unknown	11 (61.1)	1.10 (0.79–1.54)	6 (35.3)	0.99 (0.53–1.88)	9 (50.0)	0.92 (0.60–1.42)
Country of citizenship						
Guinea	**217 (57.6)**	**0.89 (0.82–0.98)**	**123 (33.0)**	**0.76 (0.65–0.89)**	220 (58.4)	0.96 (0.87–1.06)
Liberia	**19 (45.2)**	**0.71 (0.50–0.99)**	12 (29.3)	0.67 (0.41–1.08)	18 (42.9)	0.70 (0.50–1.00)
Sierra Leone	**59 (52.7)**	**0.82 (0.69–0.98)**	**31 (28.2)**	**0.65 (0.48–0.88)**	58 (51.8)	0.85 (0.71–1.02)
United States	865 (64.0)	Reference	574 (43.3)	Reference	813 (60.1)	Reference
Other/unknown	105 (70.5)	1.11 (0.99–1.24)	**82 (57.8)**	**1.34 (1.15–1.56)**	89 (59.8)	0.99 (0.87–1.14)
Total	1,265 (62.3)	NA	822 (41.3)	NA	1,198 (59.0)	NA

*RR, relative risk; NA, not applicable.
†Persons with health precautions reported as unknown are not shown. Percentages are calculated for each row. Bold indicates statistically significant associations in which the CI does not include 1.
‡Data were included only if the date of data collection was within 7 d of the date of departure from an Ebola virus disease–affected country.
§Persons who traveled for humanitarian aid, missionary, volunteer, research, or military reasons.

service-related reasons, which is consistent with previously reported data and of concern given that malaria can be a life-threatening illness (*4*). Nonetheless, a surprisingly low proportion of persons who traveled for business or service-related reasons received pretravel medical advice, used malaria prophylaxis, and received influenza vaccination. Public health agencies should work closely with organizations sending personnel abroad to improve their use of health precautions during travel. Furthermore, although most persons who traveled to visit friends or relatives received pretravel medical advice, few used malaria prophylaxis. The reason for this discrepancy deserves further evaluation.

Public health agencies should also work closely with communities whose members are likely to visit friends or relatives abroad and with medical providers caring for these communities to increase the use of travel health precautions, particularly when exceptional circumstances apply as during the EVD outbreak. Increasing the use of health precautions among persons traveling to an area for which active monitoring is recommended could directly benefit the travelers and improve the specificity of active monitoring by reducing the occurrence of malaria, influenza, and other preventable travel-associated illnesses.

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Cutaneous Leishmaniasis and Conflict in Syria

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To the Editor: War, infection, and disease have always made intimate bedfellows, with disease recrudescence characterizing most conflict zones (*1*). Recently, increasing violence from civil war and terrorist activity in the Middle East has caused the largest human displacement in decades. A neglected consequence of this tragedy has been the reemergence of a cutaneous leishmaniasis epidemic.

Old World cutaneous leishmaniasis is one of the most prevalent insectborne diseases within the World Health Organization's Eastern Mediterranean Region (*2*). Zoonotic cutaneous leishmaniasis is caused by the protozoan parasite *Leishmania major*, which is transmitted through the infectious bite of the female *Phlebotomus papatasi* sand fly; the animal reservoirs are the rodent genera *Rhombomys, Psammomys*, and *Meriones*. Anthroponotic cutaneous leishmaniasis is caused by *L. tropica* and transmitted between humans by the *Ph. sergenti* sand fly.

Until 1960, cutaneous leishmaniasis prevalence in Syria was restricted to 2 areas to which it is endemic (Aleppo and Damascus); preconflict (c. 2010) incidence was 23,000 cases/year (*3*). However, in early 2013, an alarming increase to 41,000 cutaneous leishmaniasis cases was reported (*3*,*4*). The regions most affected are under Islamic State control; 6,500 cases occurred in Ar-Raqqah, Diyar Al-Zour, and Hasakah. Because these places are not historical hotspots of cutaneous leishmaniasis, this change might be attributed to the massive human displacement within Syria and the ecologic disruption of sand fly (*Ph. papatasi*) habitats. According to the United Nations High Commissioner for Refugees, >4.2 million Syrians have been displaced into neighboring countries; Turkey, Lebanon, and Jordan have accepted most of these refugees. As a result, cutaneous leishmaniasis has begun to emerge in areas where displaced Syrians and disease reservoirs coexist (*5*).

According to the Lebanese Ministry of Health, during 2000–2012, only 6 cutaneous leishmaniasis cases were

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reported in Lebanon. However in 2013 alone, 1,033 new cases were reported, of which 96.6% occurred among the displaced Syrian refugee populations (*5*). Similarly in Turkey, nonendemic parasite strains *L. major* and *L. donovani* were introduced by incoming refugees (*6*).

Many of the temporary refugee settlements are predisposed to increased risk because of malnutrition, poor housing, absence of clean water, and inadequate sanitation. The combination of favorable climate, abundant sand fly populations, displaced refugees, and deficient medical facilities and services has created an environment conducive to cutaneous leishmaniasis reemergence. For example, refugee settlements in Nizip in southern Turkey have reported several hundred cases (*7*).

Using current datasets published in English and Arabic, we mapped cutaneous leishmaniasis prevalence within Syria and its neighboring countries (Figure). Our results demonstrate that cutaneous leishmaniasis prevalence coincides with the presence of refugee camps (Figure, panel A), which is plausible given the strong association between disease outbreaks and refugee settlements (*8*). The deterioration of Syrian health systems, including the cessation of countrywide vector control programs, has created an ideal environment for disease outbreaks (*9*). Likewise, the sand fly vectors are widely distributed throughout the Middle East; expansive *Ph. papatasi* and *Ph. sergenti* sand fly populations exist in Syria and Iraq (*4*). The presence of these vectors in regions of instability can create new cutaneous leishmaniasis foci, which might have debilitating, and often stigmatizing, consequences for residents and deployed military personnel (*10*). In addition, the distribution of *Leishmania* spp. overlaps with sand fly habitats (Figure, panel B) and disease reservoirs (W. Al-Salem, unpub. data). Consequently, the movement of large refugee populations

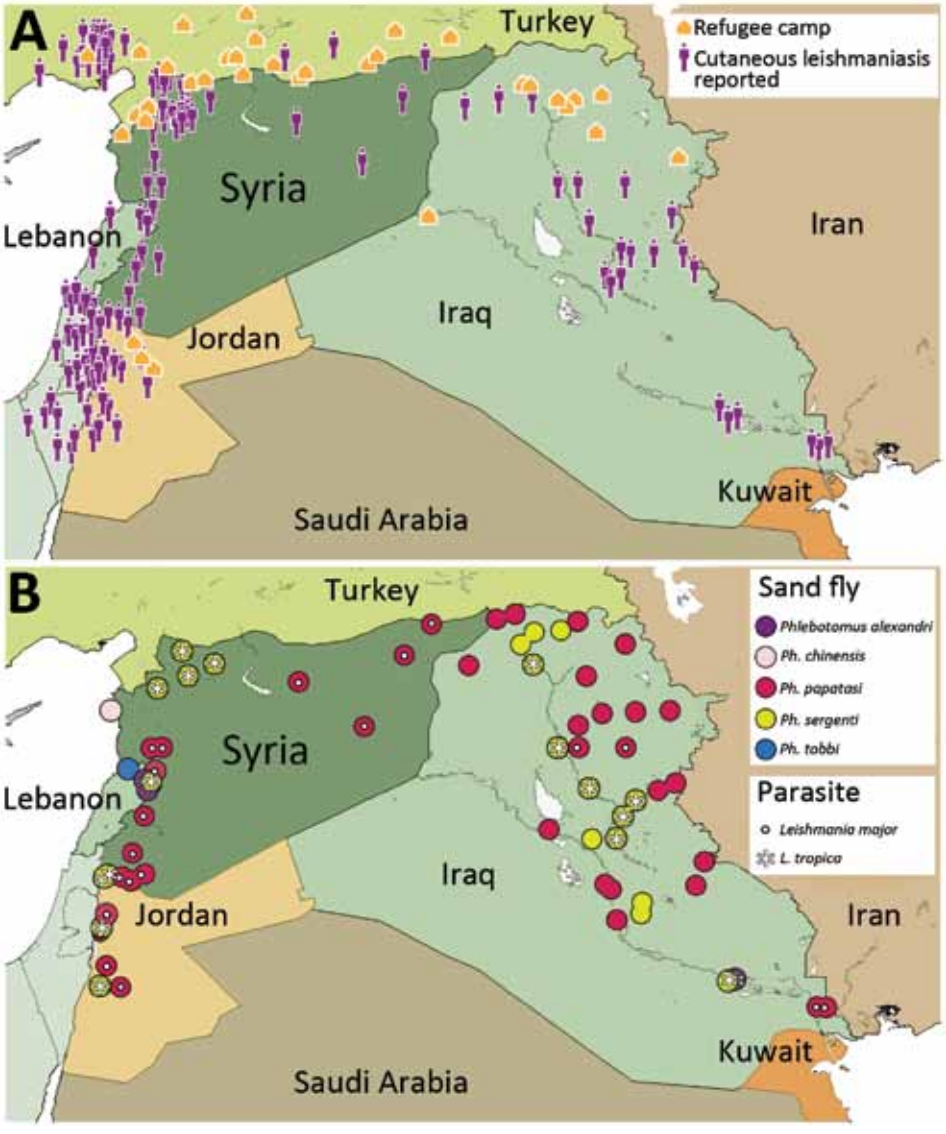

Figure. Cutaneous leishmaniasis prevalence within Syria and neighboring countries of the World Health Organization's Eastern Mediterranean Region, 2013. A) Prevalence among refugee camps. Case data were taken from http://datadryad.org/resource/doi:10.5061/dryad.05f5h. B) Distribution of sand fly and parasite species. Country names and boundaries are not official. Maps were adapted from https://hiu.state.gov/Products/SyriaDisplacementRefugees_2015Apr17_HIU_U1214.pdf.

into regions that are ill-equipped to manage imported cutaneous leishmaniasis has resulted in outbreaks in Turkey and Lebanon (*5*,*6*).

Our findings emphasize the importance of contemporaneous disease tracking to identify human populations at highest disease risk. To ameliorate the current cutaneous leishmaniasis crisis, particularly during the winter when cases start to appear, accurate disease monitoring and strategic training of persons based within refugee camps (medical staff, aid workers, volunteers, and military personnel) needs to be prioritized. Moreover, clinicians and other medical personnel residing in refugee-hosting countries must be suitably trained to diagnose cutaneous leishmaniasis because other local diseases (e.g., sarcoidosis and cutaneous tuberculosis) can have similar manifestations. Along with vector and rodent control, new cutaneous leishmaniasis outbreaks should be managed by prompt diagnosis and treatment, which are even more pertinent given that *L. tropica*–associated cutaneous leishmaniasis typically is resistant to several treatment regimens. In summary, the coexistence of sand fly populations and *Leishmania* spp. within refugee camps, together with the considerable influx of persons who already have cutaneous leishmaniasis, create a dangerous cocktail that can lead to an outbreak unprecedented in modern times.

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Phylogeny of Zika Virus in Western Hemisphere, 2015

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To the Editor: Zika virus belongs to the genus *Flavivirus*, family *Flaviviridae,* and is transmitted by *Aedes* spp. mosquitoes. Clinical signs and symptoms of human infection include fever, headache, malaise, maculopapular rash, and conjunctivitis.

Zika virus was first isolated in 1947 from the blood of a febrile sentinel rhesus monkey during a study of yellow fever in the Zika Forest of Uganda (*1*). During the next 20 years, Zika virus isolates were obtained primarily from East and West Africa during arbovirus surveillance studies in the absence of epidemics. During those 20 years, cases of Zika virus infection were detected sporadically; however, given the clinical similarity of Zika and dengue virus infections and the extensive cross-reactivity of Zika virus antibodies with dengue viruses, it is possible that Zika virus was associated with epidemics that were incorrectly attributed to dengue viruses. Beginning in 2007, substantial Zika virus outbreaks were reported first in Yap Island (Federated States of Micronesia), then in French Polynesia, and then in other Pacific Islands (*2*–*4*).

Genetic studies have revealed that Zika virus has evolved into 3 distinct genotypes: West African (Nigerian cluster), East African (MR766 prototype cluster), and Asian. It has been postulated that the virus originated in East Africa and then spread into both West Africa and Asia ≈50–100 years ago (*5*). In early 2015, cases of Zika virus infection were detected in Rio Grande State, northern

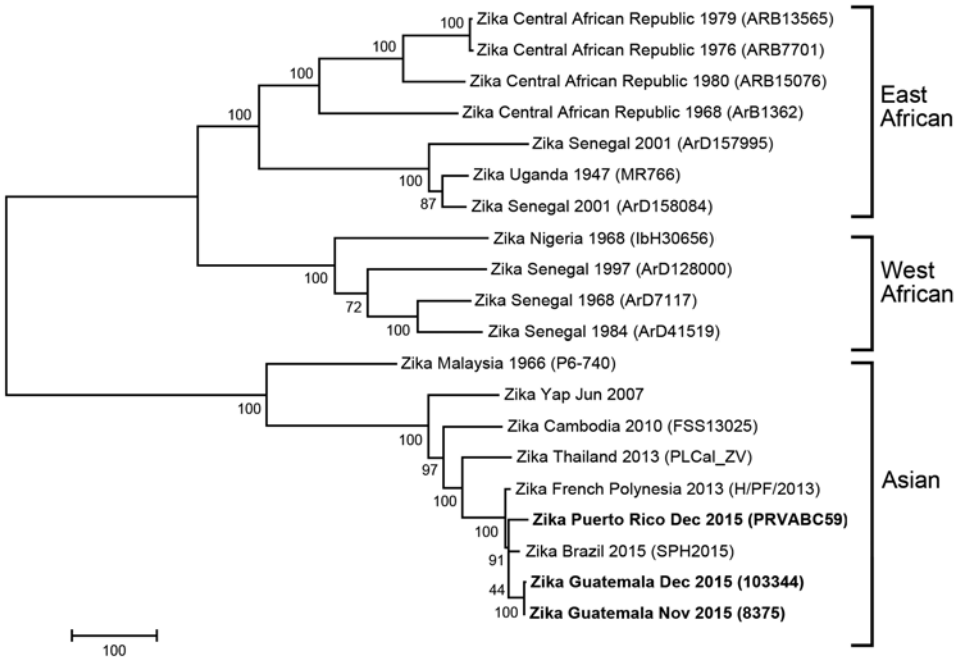

Figure. Phylogenetic tree of Zika virus isolates identified from Guatemala and Puerto Rico in December 2015 (indicated in boldface) compared with reference isolates obtained from GenBank. The isolates from Guatemala and Puerto Rico grouped with other Asian genotype viruses. The tree was derived by neighbor-joining methods (bootstrapped 1,000 times) using complete-genome sequences. Location, year identified, and GenBank strain identification for the viruses used in tree construction are shown. Scale bar indicates number of nucleotide substitutions per site. GenBank accession nos.: KU321639 (Brazil 2015 SPH2015), KJ776791 (French Polynesia H/PF/2013), KF383115 (Central African Republic ARB1362), KF383116 (Senegal 1968 ArD7117), KF383117 (Senegal 1997 ArD128000), KF383118 (Senegal 2001 ArD157995), KF383119 (Senegal 2001 ArD158084), KF268948 (CAR 1979 ARB13565), KF268949 (CAR 1980 ARB15076), KF268950 (CAR 1976 ARB7701), EU545988 (Yap 2007), KF993678 (Thailand 2013 PLCal_ZV), JN860885 (Cambodia 2010 FSS13025), HQ234499 (Malaysia 1966 P6-740), HQ234501 (Senegal 1984 ArD41519), HQ234500 (Nigeria 1968 IbH 30656), LC002520 (Uganda 1947 MR766), KU501215 (Puerto Rico PRVABC59), KU501216 (Guatemala 8375), and KU501217 (Guatemala 103344).

Brazil, and limited sequence analyses revealed that the virus was most closely related to a 2013 isolate from French Polynesia, within the Asian clade (*6*).

In December 2015, the Centers for Disease Control and Prevention Arbovirus Diagnostic Laboratory detected Zika virus in serum specimens collected from persons in Guatemala and Puerto Rico. The complete nucleotide sequence of the virus was derived directly from 3 of these serum specimens by using next-generation sequencing on the Ion Torrent (Thermo Fisher Scientific, Waltham, MA, USA) platform. The raw sequence reads were analyzed and assembled by using the CLC bio Genomics Workbench (CLC bio, Waltham, MA, USA) and Lasergene NextGen (DNAStar, Madison, WI, USA). The complete genome sequences were aligned by using ClustalW (http://www.megasoftware.net/) with all available full-length Zika virus sequences from GenBank representing the 3 genotypes. Nearly identical phylogenetic trees were generated by using several methods (minimum-evolution, maximum-likelihood, neighbor-joining), and a neighbor-joining tree was generated and analyzed with 1,000 replicates for bootstrap testing (Figure). GenBank accession numbers for sequences presented in this article are KU501215 (Puerto Rico PRVABC59), KU501216 (Guatemala 8375), and KU501217 (Guatemala 103344).

In agreement with the initial sequencing of samples from Brazil conducted by Zanluca et al. (*6*), the 3 newly sequenced Zika viruses from Guatemala and Puerto Rico are all within the Asian genotype and most closely related to strains recently isolated from Brazil (2015) and French Polynesia (2013). The tree topology confirms previous findings and indicates that Asian genotype viruses have been gradually evolving and spreading geographically throughout Asia and the Pacific Islands since at least 1966; the tree suggests that the Malaysia 1966 isolate is representative of an ancestral genotype (*7*). The percent nucleotide identity among all the Western Hemisphere Zika viruses is >99%, and as a group, these Western Hemisphere viruses are ≈89% identical (96% aa) to viruses of the East African and West African genotypes.

As reported by Musso et al. (*8*), the phylogeny and movement of Zika and chikungunya viruses are strikingly similar. Each virus is grouped into 3 genotypes of very similar geographic distribution: East Africa, West Africa, and Asia. For both viruses, it also seems that viruses from East Africa moved into Asia ≈50–100 years ago and evolved into a unique Asian genotype (*9*,*10*). In addition, the similarity with respect to the recent movement of these viruses from Asia into the Pacific Islands and then into the New World (*9*) is noteworthy. It seems that similar ecologic and/or human social factors might be responsible for the movement

of chikungunya and Zika viruses and into the New World at approximately the same time. Further studies might elucidate the exact mechanism of this transcontinental movement, leading to effective prevention strategies.

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Congenital Trypanosomiasis in Child Born in France to African Mother

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To the Editor: Sleeping sickness, or human African trypanosomiasis, is a neglected tropical parasitic infection transmitted by the tsetse fly bite. In central and western Africa, trypanosomiasis is caused by the *Trypanosoma brucei* subspecies *gambiense*. Chronic symptoms of the disease include neurologic impairment and sleep disorders (*1*). Infected children and adults can exhibit other nonspecific symptoms (*1*,*2*) attributable to biologic inflammatory syndrome, usually accompanied by an increase of IgM. Sleeping sickness can be fatal if left untreated (*3*). Although the efforts of the World Health Organization (WHO), national control programs, and nongovernmental organizations such as Médecins Sans Frontières have substantially reduced the global burden of human African trypanosomiasis, its current annual incidence is still estimated to be ≈10,000 new cases (*1*,*4*). Most cases occur during long stays in trypanosomiasis-endemic areas. Rare alternative routes of transmission are possible in nonendemic areas (e.g., through blood transfusion or organ transplantation) (*5*). We report the case of a 14-month-old boy infected with *Tr. brucei* through the transplacental route.

The child was referred to a pediatric care unit because of psychomotor retardation and axial hypotonia. His mother was from the Democratic Republic of the Congo (DRC) and had arrived in France 3 years earlier. The pregnancy, which had been initiated and monitored in France, was normal through delivery. Nonetheless, the newborn was placed with a foster family because his mother had vigilance disorders, aphasia, fluctuating hemiparesia and tetraparesia, convulsions associated with choreiform movements, anxiety, and severe depression. The foster family reported that the child did not smile and had been unable to grasp objects in the last 8 months.

Clinical examination showed z-scores of –2.25 SD and –1.38 SD for height and weight, respectively; multiple lymph node enlargement; and absence of tendon reflexes. Intermittent fever and dystonic movements were reported later during a subsequent hospitalization. The results from blood tests were compatible with chronic inflammatory syndrome (albumin 28 g/L; hypergammaglobulinemia with IgM 7.38 g/L and IgG 31.3 g/L; leukocytes 20.9 g/L; hemoglobin concentration 98 g/L). Cranial magnetic resonance

imaging showed several lesions of the white matter, mostly in the left frontotemporal lobe. The boy's cerebrospinal fluid (CSF) contained glucose at 2.5 mmol/L, protein at 0.88 g/L, and 125 leukocytes/mL (100% lymph cells). Microscopic observation of CSF and blood smear highlighted *Tr. brucei* trypomastigotes (Figure, panels A and B; Video, http://wwwnc.cdc.gov/EID/article/22/5/16-0133-V1.htm).

All the clinical and biologic findings were compatible with a diagnosis of autochthonous congenital trypanosomiasis in the meningo-encephalitic second stage. In addition to diffuse bilateral lesions of her white matter and biologic inflammatory syndrome, the mother also exhibited the same neurologic symptoms as the boy, at least since her last stay in DRC, a trypanosomiasis-endemic country that currently harbors ≈90% of new cases of African trypanosomiasis worldwide (*6*). She previously lived in Kinshasa and also reported a short visit into the bush in Angola. Until trypomastigotes were observed in her son's CSF, the mother had not received a definitive diagnosis. She was thereafter invited for clinical and laboratory examination. Lumbar puncture and blood tests were then performed: Mott cells were present in her CSF, but no parasite was detected (Figure, panel C). Diagnosis of trypanosomiasis was subsequently supported by positive serologic test results (*7*).

The 2 patients were administered 400 mg/kg eflornithine monotherapy (difluoromethylornithine) daily for 2 weeks (*8*,*9*). The clinical outcome was globally satisfactory for the mother. Her serologic tests remained positive 8 months after treatment, which is long but not unusual, and confirms that serologic testing is unreliable for posttherapeutic follow up (*4*,*7*). Magnetic resonance imaging showed a substantial decrease of her brain lesions. Her son still exhibited serious neurologic sequelae, although trypomastigotes quickly disappeared from his CSF after treatment. He still had a restricted grip, axial hypotonia, and peripheral hypertonus 2 years later. To date, he has limited contact with his environment and is not able to stand up or eat alone.

Altogether, this case is exceptional for 2 reasons: 1) the diagnosis of human African trypanosomiasis in adults and children is very unusual in countries where the disease is not endemic (<100 cases are estimated to have occurred over a 10-year period) (*10*); and 2) congenital transmission seems to be extremely rare, even in Africa (*2*). To our knowledge, 17 probable cases of congenital trypanosomiasis have been reported to date, but only 14 were sufficiently described to confirm mother-to-fetus transmission (13 were attributed to *Tr. brucei gambiense*, and 1 was attributed to *Tr. brucei rhodesiense*, the eastern African subspecies) (*2*). Because not all official guidelines mention this route of transmission, the incidence of congenital trypanosomiasis is thought to be underestimated (*4*).

The diagnosis of human trypanosomiasis should always be considered for persons who have neurologic and sleep disorders and have spent time in sub-Saharan Africa as soon as infection is suspected. Congenital trypanosomiasis is rarely studied, and incidence data are limited; however, because this is another possible route of infection, even when infection of the fetus occurs long after infection of the mother, we believe congenital transmission occurs more often than suspected.

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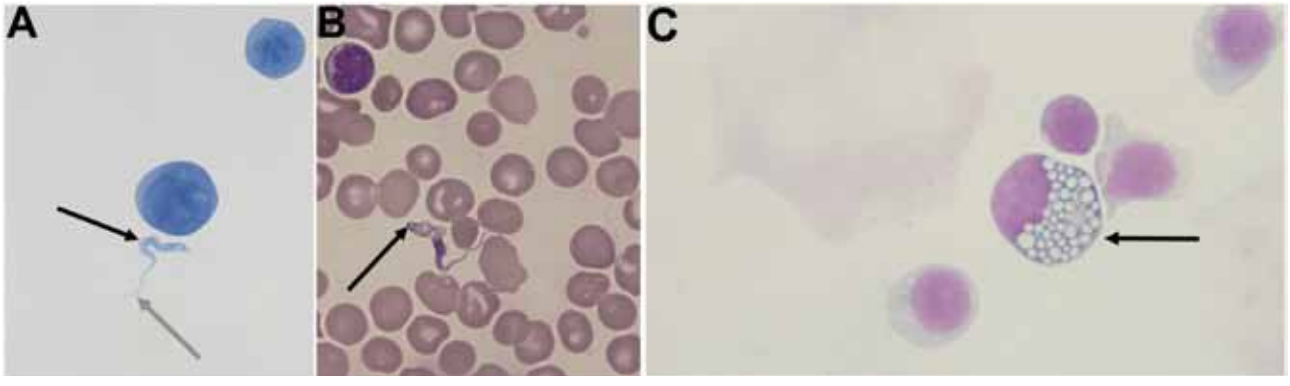

Figure. A) Cytological slide prepared from cerebrospinal fluid (CSF) from a child with congenital trypanosomiasis who was born in France to an African mother (Gram staining, original magnification ×1,000). B) Blood smear from the child (May-Grunewald Giemsa [MGG] staining, original magnification ×1,000). C) Mott cell in the mother's CSF (MGG staining, original magnification ×1,000). Trypomastigote forms of *Trypanosoma brucei* are extracellular structures, 2 × 25 μm, with a terminal flagellum (gray arrow, panel A), which is prolonged by an undulating membrane (black arrow, panel A). A central nucleus, which was difficult to visualize by Gram staining, shows that the microorganism is eukaryote. The kinetoplast (arrow, panel B) is more visible by MGG staining. It is a small organelle at the end of the cell that permits the synchronous movement of the flagellum and the undulating membrane. The kinetoplast contains circular mitochondrial DNA (*1*). The numerous mononuclear cells in CSF and blood are activated lymphocytes. The Mott cell (arrow, panel C) is a plasma cell that has spherical inclusions packed into its cytoplasm. It is often found in human trypanosomiasis (*3*). Diagnosis was subsequently confirmed by PCR. Video available online http://wwwnc.cdc.gov/eid/article/22/5/16-0133-vid1.

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Asian Genotype Zika Virus Detected in Traveler Returning to Mexico from Colombia, October 2015

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To the Editor: Zika virus is an emerging arbovirus spread by *Aedes aegypti* mosquitoes and belongs to the genus *Flavivirus* of the Spondweni serocomplex (*1*,*2*). Most often, signs and symptoms of infection are maculopapular rash, fever, arthralgia, myalgia, headache, and conjunctivitis; edema, sore throat, cough, and vomiting occur less frequently.

Zika virus is an RNA virus containing 10,794 nt, and diagnostic tests include PCRs on acute-phase serum samples to detect viral RNA (*1*). The genome contains 5′ and 3′ untranslated regions flanking a single open reading frame (ORF) that encodes a polyprotein that is cleaved into the structural proteins capsid (C), premembrane/membrane (prM), and envelope (E), and 8 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5) (*3*). Genetic studies in which nucleotide sequences derived from the NS5 gene were used indicated 3 Zika virus lineages: East African, West African, and Asian (*4*,*5*).

In Brazil, the first identified cases of dengue-like syndrome with subsequent Zika virus confirmation were documented in the early months of 2015 in the state of Rio Grande do Norte (*6*). Later that year, autochthonous transmission was reported in Colombia and Suriname during October–November (*5*) and Puerto Rico in December (*6*,*7*). During the same period, imported cases in the United States and Mexico were reported (*6*). By December 2015, we had already identified at least 15 autochthonous and 1 imported Zika cases in Mexico, initially detected by real-time reverse transcription PCR (RT-PCR). Here, we report on the documentation of a case of Zika virus infection in a male traveler returning to Mexico from Colombia in October 2015.

On October 21, 2015, we identified an imported case of Zika virus infection in the central state of Querétaro, Mexico. The patient, a 26-year-old man, had visited Santa Martha, Colombia, during the previous 12 days. Symptoms including fever, muscle pain, mild to moderate arthralgia, arthritis, back pain, chills, and conjunctivitis began on October 19, two days after his return to Mexico. A sample was collected at a primary healthcare clinic. Initial molecular testing for dengue virus at the

Queretaro Public Health Laboratory was negative; to test for Zika virus, the sample was sent to the National Reference Laboratory (InDRE), where viral RNA was extracted from it by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). We used real-time RT-PCR for diagnosis, using the Superscript III system (Invitrogen, Carlsbad, CA, USA) and primers and probes previously reported (*8*). Using Zika virus nucleotide sequence data in the Primer3Plus web interface (*8*), we amplified a 760-bp fragment with the following primers for partial characterization of viral NS5 coding gene: ZikV9113Fwd TTYGAAGCCCTTGGATTCTT and ZikV9872Rev CYCGGCCAATCAGTTCATC. We used the QIAGEN One-Step RT-PCR Kit as follows: reverse transcription at 50°C for 30 min, followed by an activation step at 95°C for 15 min and 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and a final extension step at 72°C for 10 min. We sequenced amplicons in the ABI PRISM 3130xl Genetic Analyzer instrument using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). The partial sequence of the identified strain ((MEX/InDRE/14/2015) was deposited in GenBank under accession no. KU556802.

We performed phylogenetic analysis to compare the extracted sequences with a database of 39 available nucleotide sequences from GenBank (Figure). Sequences from NS5 data were aligned, the dataset was adjusted to a common size of 531 pb, and a phylogenetic tree was constructed in MEGA6 (http://www.megasoftware.net) from aligned nucleotide sequences. The maximum-likelihood statistical algorithm and the Tamura-Nei substitution model with 1,000 replicates for bootstrap were used. Phylogenetic analyses showed that the partially sequenced strain MEX/InDRE/14/2015 belongs to the Asian lineage Zika virus and is closely related to those reported from Brazil and Suriname in 2015 (Figure). The phylogeny does show some genetic distance with respect to strains causing outbreaks in 2014 in the Americas, suggesting acquired genetic changes probably caused by adaptations during the spread of the virus, similar to those observed for chikungunya virus (*9*).

We conducted a nonsynonymous mutation analysis using the NS5 protein from the Zika virus isolated in French Polynesia in 2013 (903 aa; GenBank accession no. KJ776791.1) as a reference. The strain MEX/InDRE/14/2015bears the mutation markers K546R, K642R, and E561K, which cause the differentiation of the Asian lineage from the clades representing the African lineage (Figure). In addition, we observed that markers A527I, G588K, K531R, R648N, and S704D were acquired during the virus dispersion from Southeast Asia to the Pacific region and the Americas.

In summary, we identified Zika virus in a traveler who returned from Colombia to Mexico in October 2015.

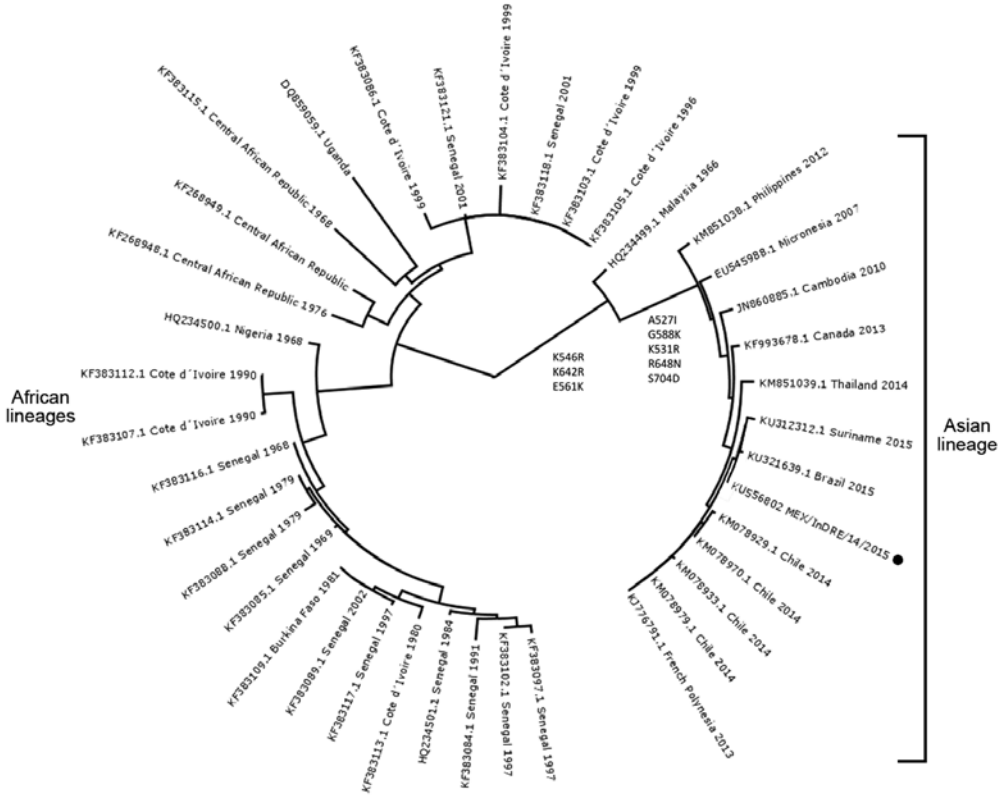

Figure. Phylogenetic analysis of nonsegmented protein 5 partial sequences of Zika virus isolated from a traveler returning from Colombia to Mexico (MEX/InDRE/14/2015; black dot), October 2015, showing close relationship Zika virus strains reported from Brazil and Suriname in 2015. We determined the evolutionary relationship implementing the maximum likelihood statistical algorithm and the Tamura-Nei substitution model using MEGA6 (http://www.megasoftware.net). The tree was created by using FigTree version 1.4.2 software (http://tree.bio.ed.ac.uk/software/figtree/). Molecular markers are indicated near the node source. Strain labels consist of GenBank accession number, country, and year of isolation.

A partial sequence of the NS5 gene showed that the Zika virus isolate from this patient was closely related to those described elsewhere in the Western Hemisphere belonging to the Asian lineage, particularly to 2 strains identified in Brazil and Suriname during 2015.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official opinion of the Ministry of Health of Mexico.

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Technological Solutions to Address Drug-Resistant *Neisseria gonorrhoeae*

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To the Editor: Since the 1930s, *Neisseria gonorrhoeae* has become resistant to drugs in every class of antimicrobial therapy used to treat it. We read with interest the article by Martin et al. about trends in Canada on *N. gonorrhoeae* susceptibility to third-generation cephalosporins, the only class of antimicrobial drugs to which most *N. gonorrhoeae* strains remain susceptible (*1*). We find the reported decrease in cefixime- and ceftriaxone-reduced susceptibility during 2010–2014 encouraging, but remain concerned about a threat from drug-resistant and untreatable *N. gonorrhoeae* infections: a similar downward trend in the United States reversed in 2014 (*2*). That divergence demonstrates the limited reliability of surveillance data.

Addressing resistance requires new methods for susceptibility determination without culture. Real-time screening for genes associated with antimicrobial drug resistance, such as *penA* mosaic alleles yielding decreased susceptibility to oral extended-spectrum cephalosporins, may be a valuable method to determine treatment (*3*). In the same issue of Emerging Infectious Diseases, Deguchi et al. described a case of multidrug-resistant *N. gonorrhoeae* (*4*), further highlighting the urgency for the innovative approach of using molecular tests to individualize treatment regimens. An ongoing study supported by the National Institutes of Health (R21AI109005) is evaluating how a laboratory-developed molecular *N. gonorrhoeae* genotypic susceptibility test for ciprofloxacin enables rapid identification of effective antimicrobial drugs (*5*).

N. gonorrhoeae may acquire new resistance mechanisms under selection pressures imposed by use of antimicrobial drugs and horizontal gene transfer from other commensal *Neisseria* species resident in the human oropharynx (*3*). Inconsistent pharyngeal *N. gonorrhoeae* screening may lead to missed opportunities for treatment. A National Institutes of Health program (Antibiotic Resistance Leadership Group, award no. UM1AI104681) is ongoing to assist manufacturers in obtaining US Food and Drug Administration approval for molecular assays to detect extragenital gonococcal infections.

For nearly 8 decades, *N. gonorrhoeae* has been controllable. Continued investment in research and the development of new laboratory technology are critical in supporting an effective response to mitigate the threat of untreatable gonorrhea.

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Detection of Zika Virus in Semen

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To the Editor: As an increasing number of autochthonous Zika virus infections are reported from several South America countries (*1*), we read with interest the report from Musso et al. on the potential sexual transmission of Zika virus (*2*). We report additional evidence for this potential route of transmission after identification of an imported case of infection into the United Kingdom.

After an outbreak alert for Zika in French Polynesia, active screening was implemented at Public Health England (Porton Down, United Kingdom). In 2014, a 68-year-old man had onset of fever, marked lethargy, and an erythematous rash 1 week after returning from the Cook Islands. Serum samples taken 3 days into the febrile illness tested negative for dengue and chikungunya viruses by real-time reverse transcription PCR (rRT-PCR). Test results for dengue virus IgM and chikungunya virus IgM also were negative; a test result for dengue virus IgG was indeterminate.

An rRT-PCR test result for Zika virus (*3*) was positive and indicated a crossing threshold value of 35 cycles. This low viral load, commonly observed even in the acute phase of disease (*3*), meant that attempts to obtain sequence data were unsuccessful. Convalescent-phase serum, urine, and semen samples were requested; only semen was positive for by rRT-PCR, at 27 and 62 days after onset of febrile illness. These results demonstrated stronger signals than those obtained in tests of the original serum sample, with crossing threshold values of 29 and 33 cycles, respectively. Zika virus–specific plaque reduction neutralization test results were positive on convalescent-phase serum samples.

Although we did not culture infectious virus from semen, our data may indicate prolonged presence of virus in semen, which in turn could indicate a prolonged potential for sexual transmission of this flavivirus. Moreover, these findings could inform decisions regarding what control methods are implemented and which specimen types are best suited for diagnostic detection.

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Claude Monet (1840–1926), *Chrysanthemums*, 1897. Oil on Canvas, 51 3/16 in x 39 in / 130 cm x 99 cm. Public domain digital image (copyright expired). Private Collection. (Painting on public display at the Cleveland Museum of Art, Cleveland, Ohio, and the Royal Academy of Arts, London during 2015–2016.)

Inspiration and Insecticide from the Flower Garden

Byron Breedlove and Paul M. Arguin

"Like the fires caught and fixed by a great colourist from the impermanence of the atmosphere and the sun, so that they should enter and adorn a human dwelling, they invited me, those chrysanthemums, to put away all my sorrows and to taste with a greedy rapture during that tea-time hour the all-too-fleeting pleasures of November, whose intimate and mysterious splendour they set ablaze all around me."

—In the Shadow of Young Girls in Flower
by Marcel Proust

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Perhaps because chrysanthemums bloom well into the autumn and come in an array of colors and varieties, these hardy flowers have inspired poets, writers, and artists for millennia. French Impressionist artist Claude Monet celebrated this flower in a series of four still-life paintings in 1897, including *Chrysanthemum, 1897*, selected for this month's cover art. These works marked the start of a fresh approach that foreshadowed Monet's renowned water-lily paintings, breaking from conventional arrangements of foods and flowers characteristic of his still-life paintings from the previous two decades. Art scholar John House wrote that the *Chrysanthemum* canvases are "unconstrained by still-life conventions, covered entirely by a vibrant, virtuoso display of blossoms and foliage."

In discussing the *Chrysanthemum* series, House noted that "The flowers fill the canvas, with no explicit spatial context. The blooms are arranged in clusters of varied color

and texture, placed against more shadowy foliage, which allows their forms to float across the whole picture surface." Art scholars Robert Gordon and Andrew Forge also commented on Monet's composition of these paintings, stating that Monet "comes at [the flowers] head on, without a compositional attitude: they are dumped in front of him, bushy or svelte, vivid, teeming with their specific energy, without atmosphere, an explosion."

"Like the fires caught and fixed by a great colourist," Monet's yellow and red flowers heighten this sense of energy, contrasting with the mass of paler white, pink, and lavender flowers. Clustered on stalks with blue-green leaves, the teeming blossoms are viewed from above, as though from an airborne pollinator's perspective. These chrysanthemums were almost certainly cultivated in the artist's own legendary gardens at Giverny. During the past 33 years of his life, Monet devoted large amounts of his time and resources to creating and sustaining his gardens, and as one critic remarked of Monet in 1898, "He reads more catalogues and horticultural price lists than articles on aesthetics."

During Monet's lifetime, chrysanthemums were relative newcomers to Europe, having not been introduced to the Western world until the 17th century. According to the National Chrysanthemum Society, in 1753 "Swedish botanist Karl Linnaeus, combined the Greek words chrysos, meaning gold with anthemon, meaning flower. . . an accurate description of the ancient species, as it also points out the mum's need for sunlight."

Images that resemble today's chrysanthemums are found on ancient Chinese pottery from as far back as the 15th century BCE. Those images, along with writings from that time, reveal that the Chinese have long cultivated *chu*, the ancient Chinese name for chrysanthemum, as a flowering herb. During the 8th century ACE, the chrysanthemum appeared in Japan, where it was called *kiku.* This flower has unusual significance for the Japanese, who adopted a single flowered chrysanthemum—a 16-floret variety called *ichimonjiginu*—as the crest and official seal of their Emperor and celebrate chrysanthemums in an annual Festival of Happiness.

Valued for more than its visual beauty, the chrysanthemum was originally imported to Japan as medicine. According to The National Chrysanthemum Society, its roots were boiled to create a tonic to relieve headaches; its sprouts and petals were added to salads; and its leaves were steeped to make a drink. The insecticidal and insect-repellent properties of some types of chrysanthemums have been recognized for thousands of years. In particular, the Dalmatian chrysanthemum, or *Tanacetum cinerariifolium,* is an important source of the natural botanical insecticide, pyrethrum.

Pyrethrum, a naturally occurring mixture of chemicals found in certain chrysanthemum flowers, kills ticks and insects such as fleas and mosquitos by attacking their nervous systems. Six individual chemical compounds called pyrethrins have active insecticidal and acaracidal properties in the pyrethrum extract.

Pyrethroids are man-made chemicals similar in structure to the pyrethrins but with more useful attributes, including increased toxicity to insects and environmental stability. Those qualities enable their use in products varying from mosquito coils and vaporizers to human and animal medicines that have household, agricultural, and public health applications. Synthetic pyrethroids such as permethrin can be applied to materials to create insect- and tick-repellent clothing and uniforms for preventing infections such as Zika virus disease, leishmaniasis, and African tick bite fever among people, including international travelers, members of the military, or public health workers.

When used as the active ingredient on long-lasting insecticide-treated bed nets hung over the sleeping spaces of persons at risk for vectorborne infections such as malaria, pyrethroids act as a repellent that reduces the numbers of insects that enter the dwelling and as an insecticide that kills insects that come into contact with the net. These mosquito nets, a cornerstone of malaria control programs worldwide, have contributed to recent dramatic reductions in the numbers of cases and deaths.

The life-saving effectiveness of the mosquito net intervention, however, is in danger of being lost, in part, because of the emergence and spread of pyrethroid resistance among mosquitoes. Developing new insecticides and resistance management strategies for their use are essential for continuing public health efforts to achieve and support the 2016 World Malaria Day theme, "End Malaria for Good."

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Article Title

Article Title: *Rickettsia parkeri* Rickettsiosis, Arizona, USA

CME Questions

1. You are evaluating a 33-year-old woman who presents with an ulcer on her left leg. She removed a tick from this site 5 days ago, and she is worried that the area is infected. You consider whether she has an infection with *Rickettsia parkeri*. Which of the following is a characteristic of *R. parkeri* infection in the current case studies?

A. Ulcerations of tick bite areas are rare
B. Skin lesions are preceded by fever
C. Systemic symptoms are common
D. Fever usually persists for 4 to 7 days after the initiation of antibiotics

2. What should you consider regarding different rickettsial infections as you evaluate this patient?

A. Conventional testing for immunoglobulin M (IgM) can differentiate *R. parkeri* from *R. rickettsii*
B. Conventional testing for immunoglobulin G (IgG) can differentiate *R. parkeri* from *R. rickettsii*
C. Advanced molecular testing can differentiate *R. parkeri* from *R. rickettsii*
D. Infection with *R. parkeri* is associated with higher rates of mortality compared with *R. rickettsii* infection

3. Which of the following findings was noted on serologic testing of patients in the current study?

A. No IgM response to rickettsial antigens at any point during testing
B. No IgM response to rickettsial antigens following the second tick bite of patient 1
C. A stronger IgG response against *R. rickettsii* antigens vs *R. parkeri* antigens
D. Negative molecular testing results for rickettsial infection

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Spectrum of Viral Pathogens in Blood of Malaria-Free Ill Travelers Returning to Canada

CME Questions

1. Your patient is a 42-year-old man with fever and malaise on returning home to Canada after travel to Latin America. Testing results are negative for malaria. According to the study by Kariyawasam and colleagues, which of the following statements about the prevalence of specific common and emerging viral pathogens in malaria-negative specimens from ill returning Canadian travelers is correct?

A. More than 10% of malaria-negative specimens were positive for common and emerging viral pathogens
B. Dengue was not detected in these specimens
C. Cytomegalovirus was the most prevalent virus detected in these specimens
D. Real-time polymerase chain reaction (PCR) revealed positivity for herpes simplex viruses 1 or 2 in 1.6%

2. According to the study by Kariyawasam and colleagues, which of the following statements about issues regarding the detection of specific common and emerging viral pathogens in malaria-negative specimens from ill returning Canadian travelers is correct?

A. Increasingly sophisticated diagnostics, such as next-generation sequencing, may be needed to close the substantial diagnostic gap and identify the full spectrum of responsible pathogens
B. Real-time PCR is helpful only when high viral load is present in peripheral blood
C. Real-time PCR has high specificity but low sensitivity
D. Most testing done for fever after travel relies on multiple pathogen detection assays

3. According to the study by Kariyawasam and colleagues, which of the following statements about clinical issues regarding specific common and emerging viral pathogens detected in malaria-negative specimens from ill returning Canadian travelers is correct?

A. Herpesvirus positivity rates in this study reflect baseline prevalence rates in the general population and isolated latent infection
B. Human herpesviruses can all cause a "mononucleosis"-like syndrome, with fever, malaise, lymphadenopathy, and mild biochemical hepatitis
C. Dengue has not been reported in the Americas
D. Chikungunya is likely to cause severe disease, whereas dengue is more likely to cause chronic arthropathy

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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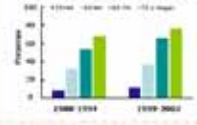

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EMERGING INFECTIOUS DISEASES®

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

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